REMARKS

Claims 48-50, 53, 64-78, 80-88, 91-93, 96-98, 100-103, 105, 106, 108-110, 114-116 are pending in this application. The Applicants have cancelled claims 51-52, 62, 63, 104, 107, and 111 without prejudice. The Applicants have amended claims 50, 53, 65-68, 72, 75, 85, 86, 91, 92, 100, and 101. Claims 115 and 116 are new. Support for these new claims can be found in the specification, for example, at page 18, lines 23-26.

Allowed Claims

The Applicants thank the Examiner for stating that claims 48, 49, 64, 78, 80-84, 86-88, 93, and 96-98 are allowable.

Examiner Interview

The Applicants thank Examiner Schnizer for the telephonic interview of October 26, 2005 in which the written description and enablement rejections were discussed, as further described below.

Claim Objection

The Office Action states that claim 92 is objected to as depending from a rejected claim, claim 91, and would be allowable if rewritten in independent form (page 20). The Applicants have rewritten claim 92 in independent form, and request that this objection be withdrawn.

35 U.S.C. § 112, 2nd Paragraph

The Office Action alleges that claims 50 and 91 are indefinite "in the use of the term 'amino terminal truncation,'" and that "[i]t is unclear if the claims are drawn to an N-terminal fragment of SEQ ID NO:4, or to a C-terminal fragment of SEQ ID NO:4" (page 3). The Applicants have amended claims 50 and 91 to indicate that the N-terminus of the fragment is at any one of amino acids 81-139 of SEQ ID NO:4.

The Office Action alleges that claim 85 is indefinite because the term "said polypeptide produced" lacks antecedent basis (page 4). The Applicants have amended claim 85 and submit that this rejection has been overcome.

The Applicants respectfully submit that claims 50, 85, and 91 are in condition for allowance and request that these rejections be withdrawn.

New Matter

The Office Action alleges that claims 50 and 91 fail to comply with the written description requirement and that "[t]here is no support in the specification as filed for a C-terminal fragment of SEQ ID NO:4 with an N-terminus corresponding to position 140 of SEQ ID NO:4" (page 5). Because the amendments to claims 50 and 91 clarify the ambiguity alleged by the Office Action, the new matter rejection can likewise be withdrawn.

Written Description

The Office Action alleges that claims 51-53, 62, 63, 65-74, 100-110, and 112-114 lack written description. Solely to expedite prosecution, the Applicants have cancelled claims 51, 52, 62, and 63 without prejudice, reserving the right to pursue this subject matter in this or related applications. Thus, the remaining independent claim rejected under this provision is claim 53, which refers to a hybridizing nucleic acid.

The Office Action alleges at page 9:

> **Example 9 [of the Written Description Guidelines] presents a claim drawn to an isolated cDNA that specifically hybridizes under highly stringent conditions to the complement of the sequence set forth in SEQ ID NO:1, wherein said nucleic acid encodes a protein that binds to a dopamine receptor and stimulates adenylate cyclase activity. Thus the protein encoded by the claimed nucleic acid must have a specific function ... In contrast the rejected claims identify no specific receptor or activity.**

Without conceding the point, the Applicants have amended claim 53 to indicate that the polypeptide encoded by the recited nucleic acid is capable of inducing apoptosis in an HT-29 colon carcinoma cell. With this functional limitation, claim 53 is analogous to the model in

Example 9 of the Written Description Guidelines.[1] Therefore, the amendment to claim 53 overcomes the written description rejection of this claim and the claims that depend therefrom.

The Applicants have amended claims 65-68, 72, 100, and 101 so they no longer depend from claims 51 or 52, and submit that these amendments overcome the written description rejection of claims 53, 65-68, 72, 100, and 101, and of the claims that depend therefrom. For at least these reasons, the Applicants respectfully request that the rejection of claims 53, 65-74, 100-103, 105, 106, 108-110, and 114 for lack of written description be withdrawn.

Enablement

The Office Action alleges that claims 51-53, 62, 63, 65-74, and 100-114 are not enabled. As discussed above, claim 53 is directed to a hybridizing nucleic acid.[2] The Applicants have amended claim 53 to indicate that the polypeptide encoded by the claimed nucleic acid is capable of inducing apoptosis in an HT-29 colon carcinoma cell. During the telephone conference, the Examiner nevertheless alleged that this claim would not be enabled for reasons stated in the office action. The rejection is respectfully traversed. The Examiner's stated position on claims with hybridization language is found on page 15 of the Action:

> **[H]ybridization guarantees structural similarity only at the nucleic acid level, and not at the polypeptide level. For example, a hybridizing nucleic acid can be 99% percent identical to SEQ ID NO:3, but if it contains a frameshift mutation in the second codon, then it does not encode a polypeptide with any structural or functional resemblance to SEQ ID NO:4. The specification as filed does not teach how to use proteins arising from any of the multitude of frameshift or nonsense mutations embraced by the claims.**

As an initial matter, nucleic acids with frameshifts and nonsense mutations can be used as hybridization probes as was known in the art. The claimed nucleic acids, by definition, hybridize to the complement of a coding sequence for human TRELL (nucleotides 106-852 or 241-852 of SEQ ID NO:3), and thus can be used to detect whether human TRELL is expressed. Thus, one skilled in the art would know how to use the claimed nucleic acids even if they had frameshift or nonsense mutations.

---

[1] available at http://www.uspto.gov/web/offices/pac/writtendesc.pdf.

[2] As discussed above, the claim here closely resembles Example 9 of the Written Description Guidelines. Although enablement and written description are different inquiries, it is unlikely that the Patent Office would illustrate its view of the patent laws with a hybridization claim if it thought that such a claim could not also be enabled.

In any event, claim 53 as presently amended refers to nucleic acids that encode a polypeptide that is capable of inducing apoptosis in an HT-29 colon carcinoma cell. At the time of the priority filing, the triplet nature of the genetic code and the three eukaryotic stop codons were described in most molecular biology textbooks and were rudimentary knowledge for those skilled in the art. For example, those skilled in the art would easily perceive a drastic frameshift and would recognize and discard nucleic acids that were so crippled.

Moreover, undue experimentation would not be required to prepare a nucleic acid according to claim 53. The level of skill in molecular biology at the time the invention was made was high, and methods of altering nucleic acids were routine. The specification at pages 18-24 mentions, and cites references regarding, PCR mutagenesis, saturation mutagenesis, degenerate oligonucleotides, alanine scanning mutagenesis, cassette mutagenesis, and other techniques. Thus, there was no impediment to creating variant nucleic acids.

Nor would one skill in the art need to engage in undue experimentation to test whether a polypeptide encoded by the altered nucleic acid recited in the claims induces apoptosis in an HT-29 colon carcinoma cell. The Office Action alleges at pages 18-19

> **In Wands, the court found that it was not undue experimentation to screen hundreds of thousands of hybridomas for a single antibody because such was routine in the art at the time of the invention. There is no evidence of record that it was routine in the art at the time of the invention to screen large numbers of mutants for TRELL activity, and the specification discloses no high throughput assay for detecting such claimed activities ...**

First, patent law does not require that claims to nucleic acids be supported by a high throughput assay to be enabled. *Wands* itself declared that extensive experimentation is permissible and did not turn on assay throughput.

Second, the application describes and provides an example (at pages 36-37) of how to test TRELL polypeptides for this function. The disclosed assay was routine at the time of filing. The fact that a routine assay for activity is provided is sufficient to find enablement under *Wands* and it is inappropriate for the Examiner to require a higher standard than that required under the law.

Third, even if the law required a high-throughput assay for enablement (which it does not), the application's exemplary assay was known to be amenable to high throughput format. As described on page 37 of the application in Table 2 and its legend, cytotoxicity was measured

by a proliferation assay (Table 2, footnote a). As described in the text on page 36, "Cell growth [proliferation] assays were carried out as previously described (Browning and Ribolini, 1989 [*J. Immunol.* 143:1859-1867])." The Browning and Ribolini paper (Exhibit A) describes cytolytic and cytostatic assays on page 1860, "Growth was quantitated by using mitochondrial reduction of the dye MTT as described ... The OD at 570 nM was determined with an ELISA plate reader" (emphasis added). Because this assay can be analyzed using an ELISA plate reader, it clearly can be used for a high throughput screen. Even in 1983, the MTT assay was known to be useful as a high throughput assay (Mosmann, *J. Immunol. Methods* 65:55-63 (1983); Exhibit B). As stated in the Abstract of this paper, "The results can be read on a multiwell scanning spectrophotometer (ELISA reader) and show a high degree of precision. No washing steps are used in the assay. The main advantages of the colorimetric assay are its rapidity and precision ..." (emphasis added). Rossi and Zetter (*Proc. Natl. Acad. Sci.* 89:6197-6201 (1992); Exhibit C) described using the MTT assay and state on page 6198, "the absorbance at 600 nm was detected using a Dynatech automated microplate ELISA reader" (emphasis added). Thus, the MTT assay can be performed in an automated high throughput mode. Clearly, at the time of filing, one skilled in the art could, without undue experimentation, use the cytotoxicity assay described in the application to determine if a nucleic acid hybridizing to the complement of a coding sequence for human TRELL (nucleotides 106-852 or 241-852 of SEQ ID NO:3) encodes a protein that induces apoptosis in an HT-29 colon carcinoma cell. Accordingly, even if the Examiner's own heightened requirement, which is not based on law or regulation, for a high-throughput assay were applied here, the present claims would pass the test.

In sum, one skilled in the art could make and use nucleic acids within the scope of claim 53. Accordingly, the Applicants respectfully request that the enablement rejection of claims 53, 65-74, and 100-103, 105, 106, 108-110, and 114 be withdrawn.

35 U.S.C. § 102

The Office Action has rejected claims 75-77 as being anticipated by Shirai et al. (U.S. Patent No. 4,921,698) because Shirai et al. teach a method of expressing a polypeptide comprising the sequence Gln-Asp-Pro in a mammalian cell. The Applicants have amended claim 75 to indicate that the polypeptide of SEQ ID NO:4 or a soluble fragment thereof that is

capable of binding to a HT-29 colon carcinoma cell and inducing apoptosis in said carcinoma cell is expressed by the method recited in the claim. The Applicants request that this rejection be withdrawn because Shirai et al. do not teach expression of the polypeptide of SEQ ID NO:4 or a soluble fragment thereof that has the recited function.

CONCLUSION

The Applicants respectfully submit that all claims are in condition for allowance, which action is expeditiously requested. The Applicants do not concede any positions of the Examiner that are not expressly addressed above. All amendments and cancellations are made without prejudice and disclaimer and may be made for reasons not explicitly stated or for reasons in addition to ones stated.

The Applicants request that the Examiner call the undersigned if helpful to expedite prosecution.

No fees are believed due. Please apply any charges or credits to deposit account 06-1050, referencing Attorney Docket No. 10274-107001.

Respectfully submitted,

Date: 2 Dec 2005

Ramon K. Tabtiang
Reg. No. 55,658

Fish & Richardson P.C.
225 Franklin Street
Boston, MA 02110
Telephone: (617) 542-5070
Facsimile: (617) 542-8906
21184795.doc

0022-1767/89/1436-1859$02.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 143, 1859-1867, No. 6, September 15, 1989
Printed in U.S.A.

STUDIES ON THE DIFFERING EFFECTS OF TUMOR NECROSIS FACTOR AND LYMPHOTOXIN ON THE GROWTH OF SEVERAL HUMAN TUMOR LINES

JEFFREY BROWNING[1] AND ANN RIBOLINI

From the Department of Cell Biology and Immunology, Biogen Inc., 14 Cambridge Center, Cambridge, MA 02142

The relative ability of TNF and lymphotoxin (LT) to inhibit the growth of five human tumor cell lines was examined both in the presence and absence of IFN-γ. Two adenocarcinoma lines, HT-29 and SK-CO-1, were 20- and 320-fold more sensitive to the inhibitory effects of TNF than LT in 3- to 4-day proliferation assays. In contrast, the breast carcinoma line BT-20 showed only a one- to twofold difference. The MCF-7 and ME-180 cell lines exhibited intermediate behavior. These results parallel the reported disparate potencies of TNF and LT in their effects on endothelial cells, hematopoietic development and their abilities to sustain a mixed lymphocyte response. Radiolabeled TNF binding studies showed two classes of receptors (K_d 0.04 to 0.15 nM and 0.2 to 1.0 nM) on the highly sensitive SK-CO-1 line. HT-29 cells also appeared to possess some high affinity-binding sites, whereas the BT-20 line completely lacked the high affinity form. Thus the presence of high affinity-binding sites correlated with increased sensitivity to the antiproliferative effects of TNF. Cold TNF competed with the binding of radiolabeled human TNF three- to fivefold better than LT for binding to all three lines. These relatively small differences between the TNF and LT receptor-binding characteristics are insufficient to explain the dramatic disparity in their antiproliferative properties. Likewise, the absolute concentrations of the unlabeled cytokines necessary to block the binding of ^{125}I-TNF were 10- to 150-fold higher than the levels necessary to elicit the biologic response. Thus, the receptor binding data conflict with the growth inhibitory effects. This discrepancy is discussed in terms of either separate receptors for TNF and LT or more complex phenomena such as receptor cooperativity possibly resulting from multivalent interactions with the trimeric form of TNF.

TNF and LT[2] also called TNF-α and TNF-β, respectively, are two proteins characterized originally for their cytolytic properties and, in the case of TNF, its in vivo tumor necrotic activity (for reviews see References 1 to 5). These proteins can be directly cytolytic to certain tumor lines, yet in other cases they can actually promote growth (6, 7). TNF is produced by many cell types including monocytes, T cells, NK cells (1, 9), and even cells of nonhematopoietic origin (8, 10), whereas LT appears to be released primarily by lymphocytes (2, 11-13). More recently, the cloned gene products have been demonstrated to display a wide range of activities. TNF appears to play major roles in specific aspects of metabolic control, the response to endotoxin shock (14), the control of hematopoietic cell development (15-18), and it shares with IL-1 many proinflammatory actions (e.g., Reference 19). Although many of these activities have also been demonstrated for LT, a comprehensive picture of their relative roles remains unclear.

Initially TNF and LT were described as having similar cytotoxic activities (20), however, more recent work has indicated that LT is less active on certain tumor lines (21, 22). A comparison of LT and TNF activities reveals disparate potencies in several systems. The human endothelial cell responds to TNF stimulation by secreting IL-1 and CSF, by expressing surface adhesion proteins, and by exhibiting increased adherence for neutrophils (23, 24). Similarly, granulocyte CSF release from fibroblasts was induced differently by LT and TNF (25). In each of these cases, LT was at least 100-fold less active than TNF. In our experiments, TNF was 20- to 50-fold more effective than LT in its ability to induce primary human umbilical vein endothelial cells to bind retinoic acid-induced HL-60 cells (J. Browning and P. Lawton, unpublished results). Likewise, LT is less effective relative to TNF in inducing human peripheral monocytes to release CSF-1 (18) and in its ability to promote the mixed lymphocyte response (26). In contrast to this pattern, LT has been reported to cause an inflammatory reaction in normal skin whereas TNF was inactive (27). In receptor-binding experiments with ME-180 (28), L929 (29, 30), HL-60 (31), and U937 (32, 33) cells, LT competed with labeled TNF almost equally well for binding to its receptor and vice versa, suggesting that both molecules interact with the same or similar receptors. The conflicting nature of the observations that these proteins share common receptors yet display widely differing activity profiles poses an interesting problem.

In this paper, we examined the anti-proliferative effects of TNF and LT on several human tumor lines both in the presence and absence of the synergistic factor, IFN-γ and have attempted to correlate differing biologic activities with receptor-binding properties. The apparent discrepancy between the receptor-binding studies and the biologic effects is discussed.

Received for publication January 25, 1989.
Accepted for publication June 19, 1989.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Correspondence and reprint requests should be addressed to Jeffrey Browning, Biogen, 14 Cambridge Center, Cambridge, MA 02142.

[2] Abbreviations used in this paper: LT, lymphotoxin; MTT, 3(4,5-dimethylthiazol-2-yl)2,5 diphenyl tetrazolium bromide; EGS, ethylene glycol bis(succinimidylsuccinate); CHO, Chinese hamster ovary.

MATERIALS AND METHODS

Cytotoxins. Human rTNF was expressed in *Escherichia coli* and purified to homogeneity as previously described (34). This preparation had a specific activity of 5.3×10^7 U/mg as defined by reference to the National Biological Standards Board TNF (Hertfordshire, England) in the 1-day L929 cell cytolytic assay. Early experiments with murine rTNF utilized protein produced at Biogen, whereas later experiments employed material that was a gift of Suntory Pharmaceutical Co. (Osaka, Japan). Both preparations had a specific activity of 3×10^7 U/mg in the L929 assay system. A cDNA clone encoding human LT was isolated from a RPMI 1788 cDNA library by using an exact oligonucleotide probe to the sequence previously described (20). The gene was stably transfected into CHO cells and amplified by using a dihydrofolate reductase/methotrexate selection system. rLT was purified from serum-free, LT-transfected CHO cell-conditioned media with a series of Sepharose S, lentil lectin, and FPLC Mono Q column chromatography steps. rLT purified by this route was 90 to 95% pure, was glycosylated, and had a specific activity of 3.1×10^7 U/mg by reference to the standard TNF sample. The protein content of the TNF and LT samples was determined by parallel amino acid composition analysis. Aliquots of cytotoxins at 1 (LT) and 10 (TNF) μg/ml were frozen and fresh aliquots thawed for each assay. Human rIFN-γ was produced from *E. coli* with a specific activity of 1.5×10^7 U/mg (encephalomyocarditis virus/WISH cell system). Murine rIFN-γ was produced in *E. coli* yielding a preparation with a specific activity of 1×10^7 U/mg by using a vesicular stomatitis virus/L929 assay system.

Cytolytic and cytostatic assays. The murine tumor line L929 and the human lines HT-29, SK-CO-1, BT-20, ME-180 and MCF-7 were obtained from the American Type Culture Collection (Rockville, MD) and grown in RPMI 1640 supplemented with glutamine, 50 μM 2-ME, penicillin/streptomycin, pyruvate, 10 mM HEPES buffer, and 10% Hyclone FCS (L929, HT-29, and ME-180 lines) or MEM (Eagle's) supplemented as above only without HEPES and with nonessential amino acids (SK-CO-1, BT-20, and MCF-7 lines). The cells were free of mycoplasma contamination (Gen-Probe test) and periodically monitored. One-day cytolytic assays using murine L929 cells were carried out by preplating L929 cells in 96-well plates (0.05 ml/well) before use. When the cells were about 50% confluent (1 to 2 days), serial dilutions of TNF or LT in 0.05 ml with 2 μg/ml mitomycin C were added. Growth was quantitated by using mitochondrial reduction of the dye MTT as described (35). After 20 to 30 h, 0.01 ml of 5 mg/ml MTT dye was added, and after an additional 3 to 4 h, 0.1 ml of isopropanol with 10 mM HCl was added to dissolve the reduced MTT dye. The OD at 570 nm was determined with an ELISA plate reader. Cytostatic assays of 3- to 4-day duration were performed as described for the cytolytic assay except that mitomycin C was omitted. Typically, wells were seeded with 3000 to 8000 cells/well at day 0, which resulted in a 0.4 to 0.8 OD signal after 3 to 4 days of growth. The initial cell concentration was adjusted to compensate for the slow growth of lines such as SK-CO-1 and BT-20. After 3 to 4 days, growth was assessed by using the MTT dye reduction method as described above. Although the absolute concentration of cytotoxin required to give 50% inhibition varied slightly from experiment to experiment, the potency of LT relative to TNF was very reproducible. In both the growth-inhibition assays and the receptor-binding studies, a monomeric m.w. was assumed for both TNF and LT.

TNF-binding assays. rTNF was iodinated by the iodogen method as described (28) except that the following ratios of reactants were used: 2 μg iodogen, 10 μg TNF, and 1 mCi of ^{125}I in a volume of 0.05 ml. ^{125}I was reacted with iodogen alone for 2.5 min at 0°C followed by the addition of TNF and the reaction continued for 10 min. The free iodine was separated on a BioRad P-6DG column. Bioassay using L929 cytotoxicity showed typically retention of at least 50% of the starting activity yielding a specific activity of 12 to 20 μCi/μg active TNF. SDS-PAGE and autoradiography showed that only TNF was iodinated. For direct binding experiments, cells from nonconfluent plates were removed with 5 mM EDTA in calcium/magnesium-free PBS, collected and resuspended at a concentration of 0.5 to 2×10^7 cells/ml by repeated passage through a 5-ml pipette into RPMI 1640 with 10% FCS and 0.1% sodium azide. Aliquots of 0.5 ml were rocked in a 3-ml conical polypropylene tube for 4 h at 4°C in the presence of ^{125}I-TNF. Nonspecific binding was assessed by including 4 μg/ml cold TNF in control samples and it constituted generally 20% or less of the total binding. The cells were washed twice with 3 ml of ice-cold Dulbecco's PBS with 5% FCS and 0.1% sodium azide and counted. Direct binding was analyzed with a standard Scatchard plot. Competitive binding was analyzed in a similar fashion by using 50 pM ^{125}I-TNF in the case of HT-29 and SK-CO-1 cells and 100 pM with the L929 cells. The same unlabeled TNF and LT solutions used in the growth inhibition assays were employed in the competition assays. Competitive binding was analysed by using logit analysis whereby logit = ln (Y/1-Y) and Y is equal to percent bound (36). In all of the receptor-binding analyses, curves were fitted by eye and no attempt was made to deconvolute curvilinear Scatchard plots by more rigorous methodologies.

TNF/LT cross-linking. ^{125}I-TNF or ^{125}I-LT (prepared as described above for TNF) was diluted into 50 mM potassium phosphate buffer, pH 7.4, with 100 μg/ml BSA and with or without various detergents. Samples at final concentrations of 100 (TNF) and 50 (LT) ng/ml were treated at 0°C with 1 mM EGS (Pierce Chemical Co., Rockford, IL; a concentrated stock solution was prepared in dry dimethylformamide) or at room temperature with glutaraldehyde (Electron Microscopy Sciences, Ft. Washington, PA) at a final concentration of 0.1% v/v. After 30 min, SDS-gel sample buffer was added and the samples were boiled and analysed by SDS-PAGE and autoradiography.

RESULTS

Properties of CHO-derived human lymphotoxin. The amino acid sequence of human LT as determined from the cDNA nucleotide sequence agreed completely with the previously published sequence (20). Because the properties of rLT secreted from CHO cells have not been well described, we have partially characterized this protein. On the basis of lentil lectin binding, the bulk of the secreted LT was glycosylated. The major rLT species migrated in SDS PAGE at an apparent M_r of 20 kDa (see Fig. 1), which is greater than the 18-kDa nonglycosylated form produced in *E. coli* (20), yet smaller than the 25-kDa species originally purified from the human B-lymphoblastoid line, RPMI 1788 (37). The amino acid composition of the purified protein agreed with the expected composition. Initial batches of rLT contained two minor species of slightly higher of M_r. *N*-terminal amino acid sequencing from polyvinylidene difluoride blotted (38) samples of all three forms revealed identical *N* termini corresponding to the *N* terminus of the 25-kDa form described by Aggarwal et al. (37). The different forms probably result from heterogeneous glycosylation. A set of LT-like proteins can be immunoprecipitated from ^{35}S-methionine-labeled, phorbol ester/PHA-stimulated PBL with a rabbit antiserum raised against the purified CHO cell-derived LT (unpublished observations). In this case a ladder of presumably different glycosylated forms was observed and the predominant form secreted from CHO cells corresponded to the smallest form produced by peripheral lymphocytes. The major species released by lymphocytes migrated at 25 kDa. In an examination of three HIV-infected human T cell lines, three different LT sizes were observed (39). Apparently, most of the natural lymphocyte-derived LT is more heavily glycosylated than CHO-derived rLT. A SDS-PAGE gel comparing recombinant *E. coli*-derived TNF with rCHO cell-derived LT is shown in Figure 1. The LT preparation was 90 to 95% pure with the major impurity being a 17-kDa protein corresponding probably to the 20-kDa form described by Aggarwal et al. (37).

TNF exists as a compact trimer in solution (40, 41). Superose S6 gel exclusion FPLC chromatography in 0.1 mg/ml BSA, 0.2 mM EDTA and PBS showed that both human and murine rTNF migrated with an apparent M_r of about 45 kDa. CHO cell-derived human rLT also migrated with a M_r of 45 kDa in this system. The iodinated preparations of TNF used in the binding studies co-migrated with unlabeled TNF. Cross-linking studies were carried out to establish more rigorously whether LT is a trimer. Figure 2 shows the results of EGS and glutaraldehyde cross-linking of iodinated TNF and LT. EGS was capable of almost completely cross-linking TNF into a

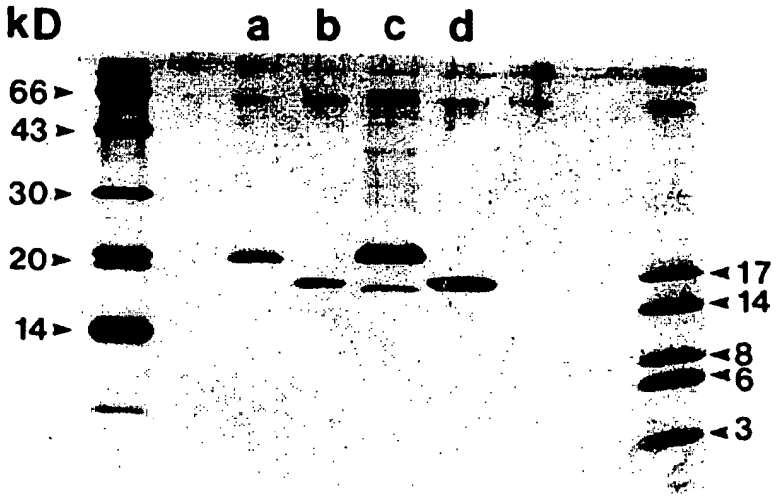

Figure 1. SDS polyacramide gel comparison of human rLT and human rTNF. Lanes were loaded with 0.2 μg (*a*, *b*) or 1.0 μg (*c*, *d*) of LT (*a*, *c*) or TNF (*b*, *d*). Gel was composed of 16% acrylamide/0.49% bis acrylamide and run according to the system described by Schaegger and Jagow (63) and stained with Coomassie blue. Bands at 68 kDa represent fingerprint artifacts.

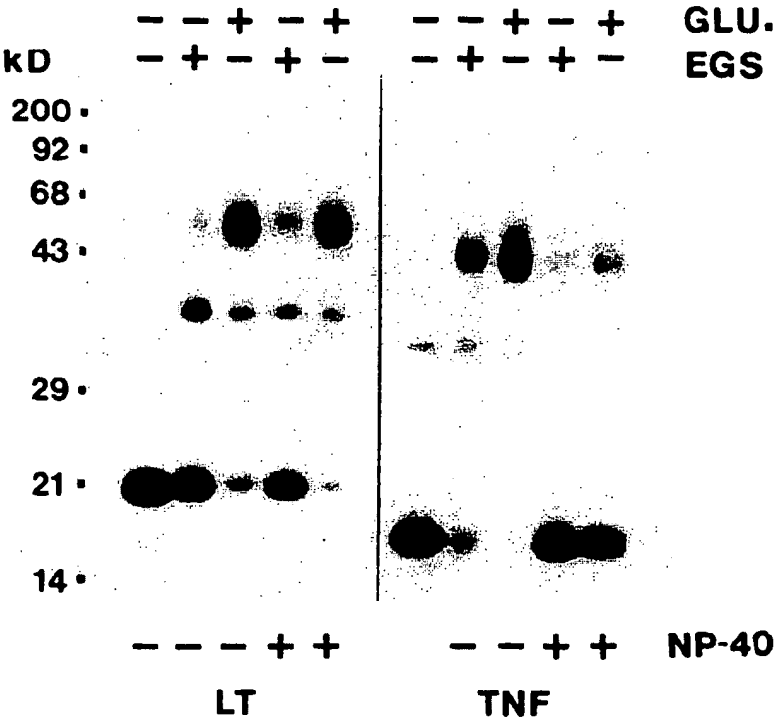

Figure 2. Reduced SDS polyacrylamide gel analysis of either EGS or glutaraldehyde (GLU.) cross-linked preparations of iodinated LT and TNF. Cross-linking was performed in the presence or absence of 1% NP-40. Shown is an autoradiograph of the gel. The m.w. standards were Bethesda Research Laboratory-prestained standards and the indicated sizes are the values as assessed by running these markers alongside the markers used in Figure 1.

trimeric structure and with LT both dimeric and trimeric forms were observed. LT is cross-linked less efficiently by EGS. Glutaraldehyde was more effective than EGS in cross-linking both TNF and LT and both glutaraldehyde-treated cytokines were found as a trimers with no indications of larger aggregates. TNF cross-linking was completely blocked by inclusion of 1% NP-40 in agreement with the reported disruption of the TNF trimeric structure by detergents (41). In contrast, the trimeric structure of LT is stable to detergent treatment. The cross-linking of LT by both EGS and glutaraldehyde was actually enhanced by addition of either 1% NP-40 or 0.1% SDS (never warmed beyond room temperature). Thus LT is a very stable trimer, even at concentrations approaching physiologic levels, and this result is in agreement with the trimeric m.w. reported for natural RPMI 1788-derived LT (42).

Specific activities of LT and TNF for cytolysis and cytostasis of L929 cells. A comparison of TNF and LT in both 1- and 3-day murine L929 proliferation assays is shown in Figure 3. LT was slightly less potent than TNF on a molar basis in the 1-day assay with 50% lysis observed at 28 to 33 pM for LT and 13 to 15 pM for TNF. On the other hand, LT was more effective in the 3-day assay (Fig. 1 and Table I) similar to results previously reported (43). The reasons for the increased potency in the 3-day assay are not clear. In the 3-day L929 antiproliferative assay, the potency of TNF also increased about twofold. The specific activity for this preparation of LT is similar to the values determined for both natural and *E. coli*-derived recombinant material as assessed by using the 1-day assay (20), thus glycosylation of the protein by the CHO cell does not affect the activity as was found previously for natural and *E. coli*-derived recombinant

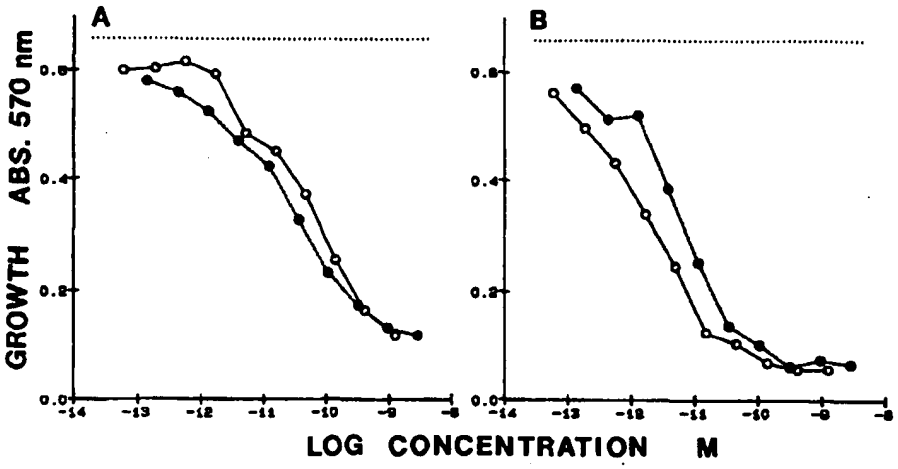

Figure 3. Comparison of recombinant human LT (O——O) and recombinant human TNF (●——●) in 1-day cytolytic (*A*) and 3-day anti-proliferative (*B*) assays using the murine fibrosarcoma L929. In the cytolytic assay about 20,000 cells/well were present, whereas in the 3-day assay, wells were seeded with 3,000 cells/well. Growth was quantitated with the MTT reduction system and relative growth is presented as OD_{570} nm. Dotted lines at the top of the panels represent the levels of growth observed in the absence of added rTNF or rLT.

TABLE I

Effects of LT and TNF on the growth of several tumor lines

Cell Line	Expt. No.	Concentration (pM) Giving 50% Growth Inhibition			
		−IFN-γ		+IFN-γ[a]	
		LT	TNF	LT	TNF
L929 (1-day)[b]	1	33	13	35	12
	2	26	15		
	3	28	14		
L929 (3-day)	1	3	12		
	2	1	4		
	3	3	8		
HT-29	1	>2500[c]	>5000	810	31
	2	>2500	>5000	372	16
SK-CO-1	1	>2500	>5000[d]	299	1
	2	>2500	>5000	300	2
BT-20	1	667	368	179	137
	2	415	285	139	106
ME-180	1	1250	184	215	13
	2	2500	552	240	33
MCF-7	1	>2500	2000	>2500	2000
	2				

[a] Murine IFN-γ was used in the case of L929 cell assays, human IFN-γ was used in all other cases. Both IFN were added at a concentration of 500 antiviral U/ml at the beginning of the assay.

[b] One-day cytolytic assay as opposed to 3-day growth assays in all other cases.

[c] The use of the greater than symbol indicates that the 50% point was not reached at this concentration.

[d] TNF stimulated growth in the absence of IFN-γ.

forms (20).

Specific activity of LT and TNF for cytostasis of several human tumor lines. IFN-γ synergizes with TNF and LT in antiproliferative assays (44–46). The effects of TNF and LT on the growth of five TNF-sensitive human tumors was examined both in the presence and absence of IFN-γ. The results of three such comparisons are shown in Figure 4 and summarized in Table I. The tumors differed dramatically both in the absolute sensitivity to TNF and LT as well as in the relative potency of these two proteins. For example, HT-29 cells have been described as being very sensitive to TNF in the presence of IFN-γ (46, 47) as was observed in these studies. In the absence of IFN-γ, HT-29 cells are unaffected by even very high levels of TNF. We had noticed during an earlier investigation of the lymphotoxin-like cytotoxins produced by the RPMI 1788 and the GM 3104 cell lines that what appeared to be natural lymphotoxin had a low specific activity on IFN-γ-treated HT-29 cells. This observation was confirmed with CHO-derived rLT in that rLT was about 20 to 25 fold less potent than TNF both on a molar basis and in terms of L929 LU. The SK-CO-1 line behaved in a fashion similar to the HT-29 adenocarcinoma in that IFN-γ was absolutely required for antiproliferative activity of LT or TNF. IFN-γ alone exhibited slight antiproliferative effects on the HT-29, SK-CO-1, and BT-20 lines at the 500 U/ml level. In the absence of IFN-γ, TNF acted as a growth factor on SK-CO-1 cells, an observation typically made in fibroblastoid systems (6, 7). Thus IFN-γ changes the way this cell interprets the signals generated by TNF, i.e., from enhanced growth to growth arrest responses. One could speculate that the antiproliferative action of IFN-γ is a result of an altered response to growth factors provided by the serum in a manner analogous to the TNF effects on the SK-CO-1 line. The antiproliferative activity of TNF was 200 to 320 times more potent than that of LT with this cell line. Although the degree of growth stimulation by TNF in the absence of IFN-γ was small at least as quantitated with the MTT assay, the concentration dependence paralleled closely that of the cytostatic activity observed in presence of IFN suggesting that IFN-γ does not modulate the affinity of the TNF receptor. A lack of receptor-affinity modulation by IFN-γ has been reported in other systems (46, 47). We attempted to block the growth inhibition of SK-CO-1 by 1 pM TNF with 50 to 100 nM LT. LT alone at these concentrations did not affect growth and its inclusion with TNF did not block the TNF activity. Hence, LT did not act to antagonize the low level TNF effect.

The breast carcinoma line, BT-20, was responsive to both LT and TNF in the absence of IFN-γ (IFN-γ being only slightly synergistic) and TNF was only one- to two-fold more potent than LT. The cell line ME-180, a cervical carcinoma, exhibited intermediate behavior with some synergistic interaction with IFN-γ and a 20-fold higher potency of TNF over LT. The breast carcinoma line, MCF-7, in our hands was relatively unresponsive to TNF with no synergy with IFN-γ. The 50% point for growth inhibition by LT for four of these lines remained in the 200- to 400-pM range. In contrast, the 50% point for TNF effects varied between 1 and 150 pM.

Receptor-binding studies. Scatchard analyses of di-

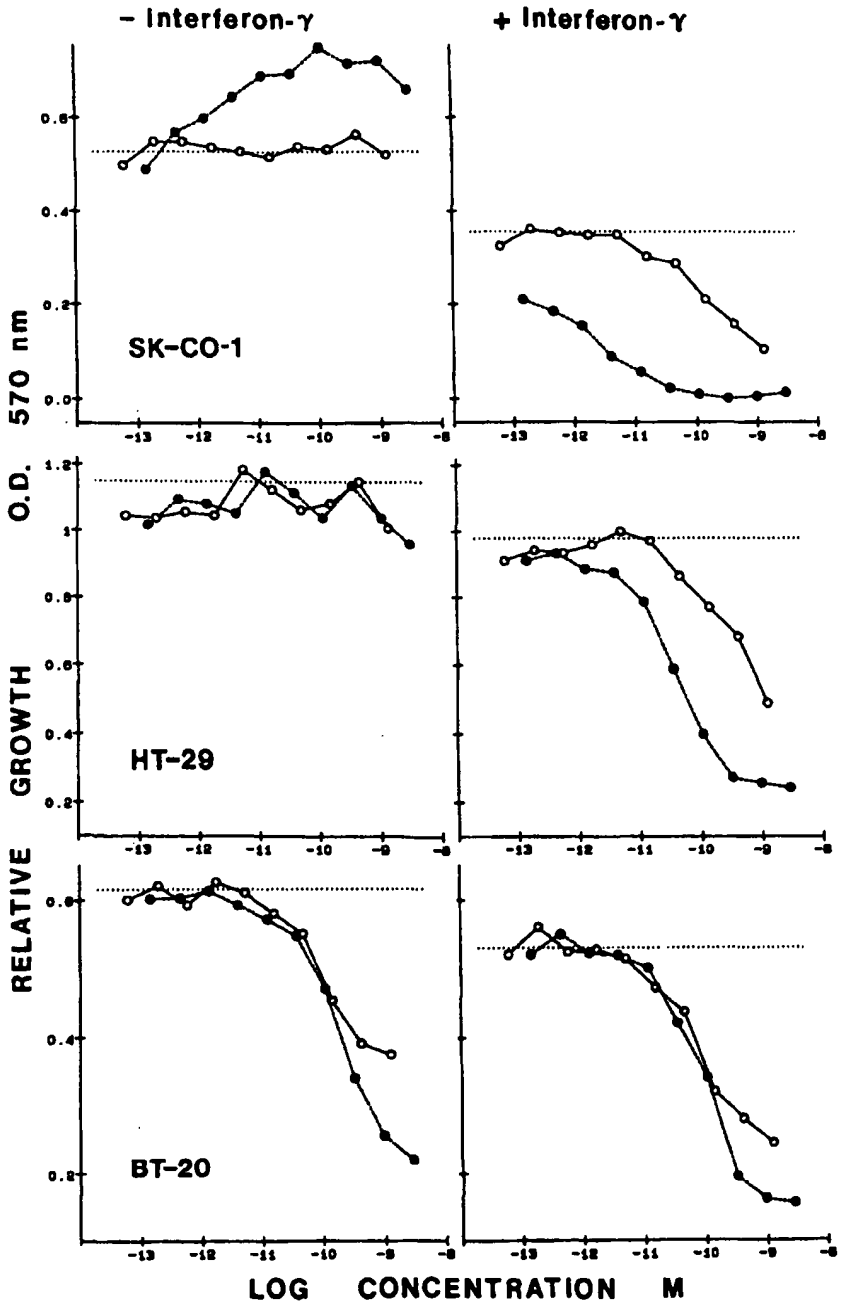

Figure 4. Comparison of the antiproliferative activity of rLT (O——O) and rTNF (●——●) on the growth of three human tumor lines: SK-CO-1, HT-29, and BT-20. Wells were seeded with 8000 (SK-CO-1 and BT-20) and 4000 (HT-29) cells/well and growth was assessed after 3 days (HT-29 and SK-CO-1) or 4 days (BT-20). Cultures were set up either with or without 500 U/ml of IFN-γ. Dotted line signifies the level of growth observed in the absence of rTNF or rLT, either with or without IFN-γ.

rect ^{125}I-TNF-binding experiments are shown in Figure 5 and summarized in Table II. In these studies, the concentration of active TNF was quantitated by using the L929 bioassay and specific activities were calculated, assuming that both inactive and active molecules were labeled uniformly. In one case the self displacement method was used to assess the sp. act. and a roughly similar value was obtained (48). The L929 cell line bound 2500 to 5500 molecules of human TNF per cell with a dissociation constant of 0.5 to 1.0 nM, a value that agrees well with previously published results (30, 49). In one set of experiments, the Scatchard plots had clearly defined concave downward appearances when data at low TNF concentrations were included (data not shown). Such plots can be indicative of positive cooperativity (50). Scatchard analysis of TNF binding to SK-CO-1 cells revealed two types of binding sites, a low affinity form with 3000 to 6000 sites per cell and a high affinity form with 1000 to 2000 sites/cell. The dissociation constant of the high affinity form, K_d 0.07 to 0.15 nM, was 5- to 10-fold higher than that of the low affinity form, K_d 0.2 to 1.0 nM. The HT-29 Scatchard plot was curvilinear, probably a result of contributions by both a low affinity site similar to that observed on the SK-CO-1 line and some higher affinity components. The number of receptor sites increased slightly with a 6-h pretreatment with IFN-γ, however, these increases were not observed with 24- to 48-h exposure paralleling previous reports (46, 47). The BT-20

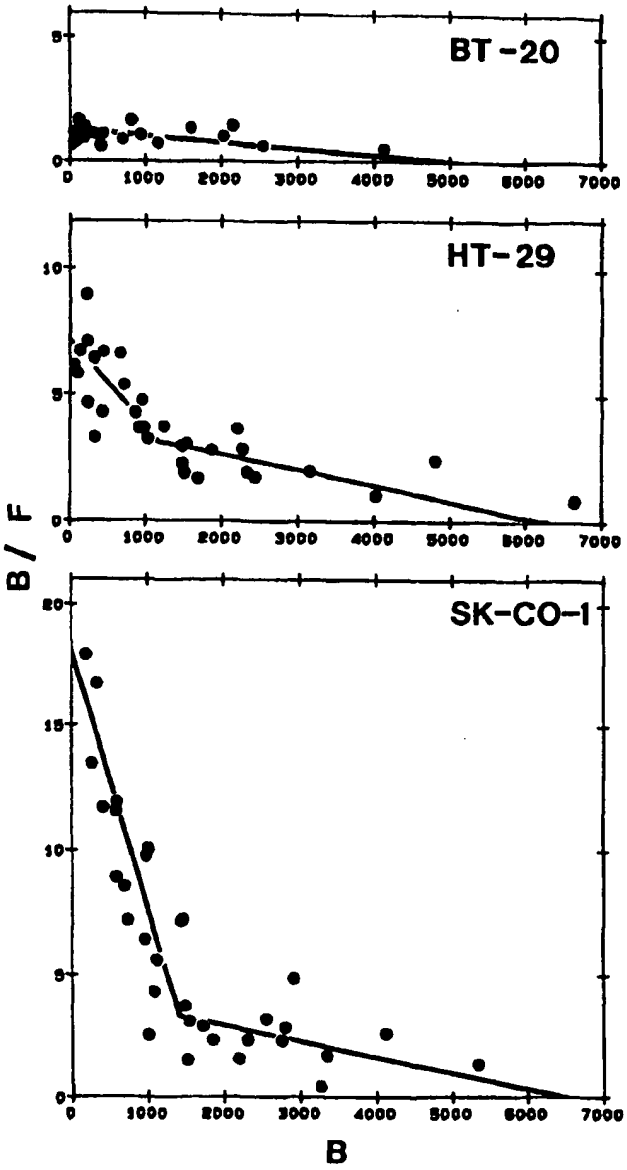

Figure 5. Scatchard analyses of the direct binding of ^{125}I-TNF to three human tumor lines. *B* represents number of TNF molecules per cell. Shown are data pooled from several experiments, and in each case the cells had been treated with IFN-γ for 6 to 48 h.

TABLE II

Summary of Scatchard analyses of direct ^{125}I-TNF binding to various tumor lines

Cell	−IFN-γ		+IFN-γ[a]	
	Sites/cell	K_d (nM)[b]	Sites/cell	K_d (nM)
L929	2500–5500	0.61–0.80		
BT-20			3000–5000	3.30–6.50
HT-29	2000–3300	0.25–1.00	1100–6000	0.40–1.10
SK-CO-1				
Low[c]	3000	0.46	3000–6500	0.20–1.00
High	1500	0.07	600–2000	0.04–0.15

[a] Pretreatment with 1000 U/ml IFN-γ for 6 or 48 h.
[b] Data represent the range of all experiments.
[c] Indicative of the high and low affinity sites found with this cell line.

line possessed the lowest affinity receptors and clearly lacked any high affinity receptor forms. Thus increased sensitivity to the biologic effects of TNF correlated with the presence of apparently higher affinity receptors.

Logit plots of pooled data from competitive binding experiments are shown in Figure 6 and the results of individual experiments summarized in Table III. Figure 6 represents data from both multiple cell preparations and different iodinated TNF lots. Both LT and TNF competed with labeled TNF binding to all the cell types examined. In the case of the murine L929 cell, human LT was similar to murine TNF and both were more effective than human TNF. Whether this increased binding of LT over TNF simply reflects aspects of the species barrier or implies that the L929 receptor is actually a LT receptor is not clear. In contrast to the murine cell line, LT was three- to fivefold less effective than TNF in competing with labeled TNF binding to the human HT-29 and SK-CO-1 lines. The BT-20 line possesses relatively low affin-

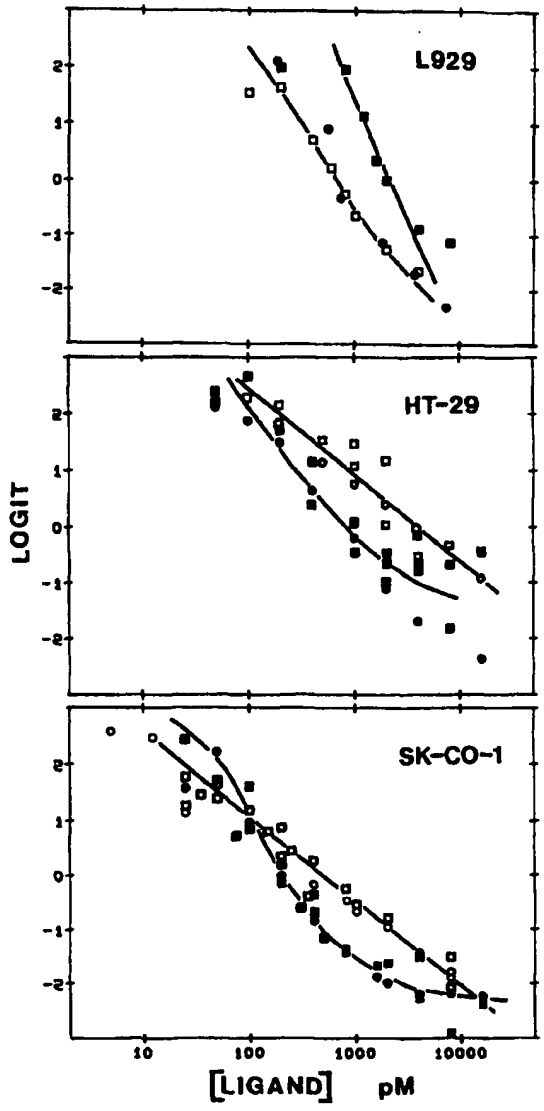

Figure 6. Logit analysis of the competition of unlabeled human rLT (*open symbols*) or unlabeled human rTNF (*closed symbols*) with ^{125}I-human TNF for binding to murine L929, human HT-29, and human SK-CO-1 cells. In the experiments with HT-29 and SK-CO-1 cells, data from several experiments have been pooled and different symbols were used to denote whether the cells were pretreated with IFN-γ (*squares*) or were untreated (*circles*). In the L929 cell panel, the *closed circles* represent competition by unlabeled murine rTNF and the cells were not exposed to IFN-γ.

TABLE III
Summary of competitive binding experiments

Cell Line	Concentration of Unlabeled Ligand (pM) at[a] Half-Maximal Inhibition			
	−IFN-γ[b]		+IFN-γ	
	TNF	LT	TNF	LT
L929	2,300	600		
BT-20			~6,000	~30,000
HT-29	1,000	3,000	1,000	2,000
SK-CO-1	180	350	180	450

[a] Concentration of unlabeled TNF or LT required to displace one-half of the bound ^{125}I-TNF from the cells.
[b] The cells were incubated with or without IFN-γ for 6 h before performing the binding assay.

ity receptors and although accurate data could not be obtained, clearly much higher levels of TNF and LT were required to displace labeled TNF from receptor sites on this cell. Interferon treatment had no effect on the ability of cold ligand to displace labeled TNF, again agreeing with the lack of modulation of receptor affinity by IFN. In these experiments, the relative binding affinities of TNF for these lines as defined by the 50% inhibition point roughly paralleled the antiproliferative effects, however, the concentrations were generally at least 10-fold higher than the level yielding 50% of the biologic effect. In a classic analysis of various agonists competing for the same receptor, the binding curves can be shifted reflecting unequal receptor affinities, yet they should be parallel. In this logit analysis, the TNF curve for the SK-CO-1 line was found to be nonlinear and had a different slope when compared with the LT binding curve. The quality of the HT-29 data do not allow one to define accurately the curve shape. This observation indicates that the interactions of these two ligands with the receptor are not identical. Looking at the TNF-binding curve with the SK-CO-1 line (Fig. 6), one could surmise that the curves were roughly parallel at high ligand concentrations, yet deviate in the low concentration regions. The nonlinearity in the TNF curve may reflect the receptor heterogeneity seen in the Scatchard analysis. It should be noted that radiolabeled TNF binding studies may be plagued by an additional problem. Iodination by methods such as Iodogen that yield high specific activity labeling do lead to TNF inactivation when the labeling is allowed to continue for longer times (J. Browning, unpublished observations). It is possible that individual subunits of the TNF trimer may be inactivated without serious loss of biologic activity, i.e., less than 50 to 70% losses. These partially defective yet more highly iodinated TNF molecules may exhibit abnormal binding in Scatchard analyses.

DISCUSSION

We have shown in this study that TNF and LT have different antiproliferative properties on several human tumor lines. The concentration of LT necessary for a half maximal antiproliferative effect remained in the range of 200 to 400 pM for three tumor lines, whereas the TNF concentrations required for similar activity varied over two orders of magnitude. With few exceptions, the presence of only one class of TNF-binding site has been demonstrated with dissociation constants ranging from 1 to 2000 pM depending upon the cell type. Unlabeled TNF and LT have been shown to compete with labeled TNF for binding to its receptor (28, 30) and vice versa, i.e., cold TNF could compete with labeled LT binding to its receptor (29, 31, 32). Typically, LT and TNF were almost equally potent in this respect. The simpliest model accommodating these data is the existence of a single receptor that can bind both LT and TNF. If one assumes that the biologic response is directly linked to receptor occupancy and that only a single type of TNF/LT receptor is present on these cells, it is difficult to explain the differing biologic effects of TNF and LT. In this case, the differing sensitivities to TNF may simply reflect the varying levels of signal transduction required to produce an effect, e.g., only 0.1 to 1% occupancy of the TNF receptors on a SK-CO-1 cell may be sufficient to trigger cytostasis. However, if LT is behaving as a simple TNF agonist, it too should trigger the biologic event at proportionately lower concentrations with a cell line like SK-CO-1. Because the potency of LT remained relatively unaltered in these different cells, more complex phenomena need to be considered.

Two possible explanations for this discrepancy have occurred to us. In the first case, both low and high affinity receptors for TNF exist with LT binding to a low affinity receptor with a K_d of about 10^{-10} to 10^{-9} M, but not to the high affinity form (K_d 10^{-10} to 10^{-12} M). In this model, signal transduction results from occupancy of either receptor form, however, the increased sensitivity of certain cell types is a consequence of TNF binding to the high affinity-receptor form. The high and low affinity forms may reflect either different states of the same receptor or different receptor molecules. In the second model, TNF binding to its receptor occurs with some form of positive cooperativity possibly stemming from its trimeric structure. The apparent high affinity binding would represent actually a high avidity binding mode of a common low affinity receptor. In this model, LT would be incapable of interacting in a cooperative fashion. Whether cooperative interactions occur would depend probably not only on the receptor density, but also on other factors such as cytoskeletal linkages, etc.

TNF-binding studies were undertaken in an attempt to resolve this dilemma and these results suggest that elements of both models may contribute. SK-CO-1 cells and probably HT-29 cells were found to possess a high affinity form of the TNF receptor correlating with the increases sensitivities of these lines to TNF. High levels of LT, that alone were inactive, were unable to affect the low level anti-proliferative effects of TNF with the SK-CO-1 line suggesting that LT cannot bind to the high affinity form. Furthermore, one could speculate that the high affinity form may be the biologically relevant TNF receptor and hence LT may not elicit TNF-like activities under physiologic conditions.

On the other hand, TNF was only three- to fivefold better than LT in competing with ^{125}I-TNF binding to the SK-CO-1 receptor in contrast to the 150- to 100-fold difference in biologic potencies. Thus the existence of a high affinity TNF receptor may explain the increased sensitivity of a cell type such as SK-CO-1 to TNF, however, the LT binding properties do not fit with its decreased biologic potency. It is possible that the competitive binding studies are complicated by the existence of a heterogenous receptor population. Although most studies have not detected high affinity TNF binding, there are

a number of exceptions. High affinity TNF binding (K_d 1 to 5 pM) was observed with human monocytes (51) and HL-60 cells (16, 52) and in the latter case the presence of the high affinity form correlated with increased TNF potency. Likewise, high affinity receptors with K_d in the 1 to 5 pM range for murine TNF were described on the mouse L cell (53). Intermediate affinity receptors in the range of 20 to 70 pM have been described on endothelial cells, lymphocytes, and the breast carcinoma line MCF-7 (23, 54–56). Evidence for multiple TNF receptors also comes from consideration of the species specificity of receptors on several murine cell lines. The murine L929 cell responds almost equally well to both human and murine TNF, yet the murine CT6 T cell line (57), the mouse/rat T cell hybridoma PC-60 (58), and the murine line PG-19 (59) respond to murine TNF but not to human TNF. Whether these phenomena reflect separate LT and TNF receptors is not clear.

The analysis of ligand binding to multiple receptors can be very complex, nonetheless, the data obtained in this study suggest an additional possibility. The logit plots of TNF and LT competition for labeled TNF binding should yield parallel lines if the two ligands interact in a similar manner with the same receptor albeit with different affinities. The lack of parallel plots may indicate some form of receptor cooperativity. Trimeric TNF could undergo multivalent interactions with TNF receptors leading to a high avidity TNF interaction. This interaction could allow for biologic effects at low concentrations despite low to moderate affinity TNF binding to its receptor. In this model, LT must be considerably less effective in forming multimeric interactions with this receptor form. In support, it was found that monomeric TNF was biologically less active and bound with a much lower affinity than trimeric TNF (41). Conversely, one study employing iodinated monomeric *E. coli* derived-human LT observed relatively high affinity binding to the L929 cell receptor (29). Although most TNF cross-linking studies have indicated the existence of a 55- to 75-kDa receptor chain, the molecular nature of the TNF receptor remains presently unresolved. Immunoaffinity-purified TNF receptor was found to undergo ligand-dependent oligomerization, possibly reflecting binding of one TNF trimer with several receptor molecules (60). An additional 138-kDa protein was associated with TNF-responsiveness in the MCF-7 carcinoma (54) and the possibility that this species resulted from TNF cross-linked dimeric forms should be considered. Resolution of these aspects awaits the cloning and characterization of the TNF/LT receptors.

LT and TNF pose a problem arising in other apparently redundant systems such as IL-1 types α and β and IFN-α and -β; namely, why do multiple forms of these cytokines exist? Although a region in the C-terminal domain that appears to be critical for receptor binding is relatively conserved (61), the amino acid sequences of LT and TNF are only 28% homologous overall (1, 2). The genes lie within 2 kb of each other (1), apparently the result of a gene duplication event, yet they are independently regulated (12, 13). Other than the disparate activities in the endothelial cell and monocyte systems and the antiproliferative effects described here, the qualitative biologic spectra of LT and TNF are quite similar. It is possible that the actual role of LT lies in an as yet undescribed system and relies on interaction with a LT-specific high affinity receptor. It was in this light that several groups have reintroduced the suggestion that LT may be a mediator of delayed type hypersensitivity reactions (26, 62). Whether nature enjoys the evolutional security of these redundant cytokines or exploits individual activities via separate receptors remains an open question.

Acknowledgments. The authors would like to thank the following people for their contributions to this work: Jerry Carson and C. Hession for isolation of the cDNA clone, J. Devlin for preparation of the RPMI 1788 RNA, R. Rothenbuhler and E. Kawashima for DNA sequencing, R. Moova for various expression constructs, J. Travernier and W. Fiers for generating the LT-transfected CHO line, E. P. Chow and E. Greco for protein sequence analyses, and B. Pepinsky and C. Ware for helpful advice.

REFERENCES

1. Goeddel, D. V., B. B. Aggarwal, P. W. Gray, D. W. Leung, G. E. Nedwin, M. A. Palladino, J. S. Patton, D. Pennica, H. M. Shepard, B. J. Sugarman, and G. H. W. Wong. 1986. Tumor necrosis factors: gene structure and biological activities. *Cold Spring Harbor Symp. Quant. Biol. 51:597.*
2. Paul, N. L., and N. H. Ruddle. 1988. Lymphotoxin. *Ann. Rev. Immunol. 6:407.*
3. Old, L. J. 1985. Tumor necrosis factor. *Science 230:630.*
4. Beutler, B., and A. Cerami. 1988. Tumor necrosis, cachexia, shock and inflammation: a common mediator. *Ann. Rev. Biochem. 57:505.*
5. Sherry, B., and A. Cerami. 1988. Cachectin/tumor necrosis factor exerts endocrine, paracrine and autocrine control of inflammatory responses. *J. Cell Biol. 107:1269.*
6. Sugarmann, B. J., B. B. Aggarwal, P. E. Hass, E. S. Figari, M. A. Palladino, and H. M. Shepard. 1985. Recombinant human tumor necrosis factor-α: effects on proliferation of normal and transformed cells *in vitro*. *Science 230:943.*
7. Vilcek, J., V. J. Palombella, D. Henriksen-Destanfano, C. Swenson, R. Feinman, M. Hirai, and M. Tsujimoto. 1986. Fibroblast growth enhancing activity of tumor necrosis factor and its relationship to other polypeptide growth factors. *J. Exp. Med. 163:632.*
8. Spriggs, D. R., K. Imamura, C. Rodriguez, E. Sariban, and D. W. Kufe. 1988. Tumor necrosis factor expression in human epithelial tumor cell lines. *J. Clin. Invest. 81:455.*
9. Turner, M., M. Londei, and M. Feldmann. 1987. Human T cells from autoimmune and normal individuals can produce tumor necrosis factor. *Eur. J. Immunol. 17:1807.*
10. Rubin, B. Y., S. L. Anderson, S. A. Sullivan, B. D. Williamson, E. A. Carswell, and L. Y. Old. 1986. Nonhematopoietic cells selected for resistance to tumor necrosis factor produce tumor necrosis factor. *J. Exp. Med. 164:1350.*
11. Nedwin, G., L. P. Sverdersky, T. S. Bringman, M. A. Palladino, and D. V. Goeddel. 1985. Effect of interleukin-2, interferon-γ and mitogens on the production of tumor necrosis factors α and β. *J. Immunol. 135:2492.*
12. Cuturi, M. C., M. Murphy, M. P. Costa-Giomi, R. Weinmann, B. Perussia, and G. Trinchieri. 1987. Independent regulation of tumor necrosis factor and lymphotoxin production by human peripheral blood lymphocytes. *J. Exp. Med. 165:1581.*
13. Sung, S-S. J., J. M. Bjorndahl, C. Y. Wang, H. T. Kao, and S. M. Fu. 1988. Production of tumor necrosis factor/cachectin by human T cell lines and peripheral blood T lymphocytes stimulated by phorbol myristate acetate and anti-CD3 antibody. *J. Exp. Med. 167:937.*
14. Cerami, A., and B. Beutler. 1986. Cachectin: the dark side of tumor necrosis factor. *Cold Spring Harbor Symp. Quant. Biol. 51:625.*
15. Roodman, G. D., A. Bird, D. Hutzler, and W. Montgomery. 1987. Tumor necrosis factor-alpha and hematopoietic progenitors: effects of tumor necrosis factor on the growth of erythroid progenitors CFU-E and BFU-E and hematopoietic cell lines K562, HL-60 and HEL cells. *Exp. Hematol. 15:928.*
16. Peetre, C., U. Gullberg, E. Nilsson, and L. Olsson. 1986. Effects of recombinant tumor necrosis factor on proliferation and differentiation of leukemic and normal hemopoietic cells in vitro. *J. Clin. Invest. 78:1694.*
17. Murphy, M., B. Perussia, and G. Trinchieri. 1988. Effects of recombinant tumor necrosis factor, lymphotoxin and immune interferon on proliferation and differentiation of enriched hematopoietic precursor cells. *Exp. Hematol. 16:131.*
18. Oster, W., A. Lindemann, S. Horn, R. Mertelsmann, and F. Hermann. 1987. Tumor necrosis factor (TNF)-alpha but not TNF-beta induces secretion of colony stimulating factor for macrophages (CSF-

1) by human monocytes. *Blood 70:1700.*
19. **Okusawa, S., J. A. Gelfland, T. Ikejima, R. J. Connolly, and C. A. Dinarello.** 1988. Interleukin-1 induces a shock like state in rabbits: synergism with tumor necrosis factor and the effect of cyclooxygenase inhibition. *J. Clin. Invest. 81:1162.*
20. **Gray, P. W., B. B. Aggarwal, C. V. Benton, T. S. Bringman, W. J. Henzel, J. A. Jarrett, D. L. Leung, B. Moffat, P. Ng, L. P. Sverdersky, M. A. Palladino, and G. E. Nedwin.** 1984. Cloning and expression of cDNA for human lymphotoxin, a lymphokine with tumor necrosis activity. *Nature 312:721.*
21. **Granger, G. A., I. Masunaka, B. Averbook, M. Kobayashi, M. Fitzgerald, and R. Yamamoto.** 1988. Differences in the bioactivity of recombinant human TNF, LT and T-cell derived LT-3 on transformed cells in vitro and the Meth A tumor growing in BALB/c mice. *Lymphokine Res. 7:488.*
22. **Bevan, M., B. S. Anderson, P. Kelleher, F. Whalen, K. McCredie, and J. Gutterman.** 1987. Diversity of effect of recombinant tumor necrosis factors α and β on human myelogenous leukemia cell lines. *Blood 69:721.*
23. **Lockaley, R. M., F. P. Heinzel, H. M. Shepard, J. Agosti, T. E. Eessalu, B. H. Aggarwal, and J. M. Harlan.** 1987. Tumor necrosis factors α and β differ in their capacities to generate interleukin-1 release from human endothelial cells. *J. Immunol. 139:1891.*
24. **Broudy, V. C., J. M. Harlan, and J. W. Adamson.** 1987. Disparate effects of tumor necrosis factor α/cachectin and tumor necrosis factor β/lymphotoxin on hematopoietic growth factor production and neutrophil adhesion molecule expression by cultured human endothelial cells. *J. Immunol. 138:4298.*
25. **Koeffler, H. P., J. Gasson, J. Ranyard, L. Souza, M. Shephard, and R. Munker.** 1987. Recombinant human TNF stimulates production of granulocyte colony-stimulating factor. *Blood 70:55.*
26. **Shalaby, M. R., T. Espevik, G. C. Rice, A. J. Ammann, I. S. Figari, G. E. Ranges, and M. A. Palladino.** 1988. The involvement of human tumor necrosis factors-α and -β in the mixed lymphocyte reaction. *J. Immunol. 141:499.*
27. **Averbook, B. J., R. S. Yamamoto, T. R. Ulich, E. W. B. Jeffes, I. Masunaka, and G. A. Granger.** 1987. Purified native and recombinant human alpha lymphotoxin [tumor necrosis factor (TNF)-beta] induces inflammatory reactions in normal skin. *J. Clin. Immunol. 7:333.*
28. **Aggarwal, B. B., T. E. Eessalu, and P. E. Hass.** 1985. Characterization of receptors for human tumor necrosis factor and their regulation by γ-interferon. *Nature 318:665.*
29. **Hass, P. E., A. Hotchkiss, M. Mohler, and B. Aggarwal.** 1985. Characterization of specific high affinity receptors for human tumor necrosis factor on mouse fibroblasts. *J. Biol. Chem. 260:12214.*
30. **Coffman, F. D., L. M. Green, and C. F. Ware.** 1988. The relationship of receptor occupancy to the kinetics of cell death mediated by tumor necrosis factor. *Lymphokine Res. 4:371.*
31. **Gullberg, U., M. Lantz, E. Nilsson, C. Peetre, G. Adolf, and I. Olsson.** 1987. Characterization of a relationship between the T-lymphocyte derived differentiation inducing factor (DIF) and lymphotoxin: a common receptor system for DIF, lymphotoxin and tumor necrosis factor downregulated by phorbol esters. *Eur. J. Haematol. 39:241.*
32. **Stauber, G. B., and B. B. Aggarwal.** 1989. Characterization and affinity cross-linking of receptors for human recombinant lymphotoxin (tumor necrosis factor-β) on a human histiocytic lymphoma cell line, U937 . *J. Biol. Chem. 264:3573.*
33. **Hirano, K., K. Yamamoto, Y. Kobayashi, and T. Osawa.** 1989. Characterization of specific high-affinity receptor for human lymphotoxin. *J. Biochem. 105:120.*
34. **Marmenout, A., L. Fransen, J. Travernier, J. van der Heyden, R. Tizard, E. Kawashima, A. Shaw, M-J. Johnson, D. Semon, R. Mueller, M-R. Ruysschaert, A. van Vlhet, and W. Fiers.** 1985. Molecular cloning and expression of human tumor necrosis factor and comparison with mouse tumor necrosis factor. *Eur. J. Biochem. 152:515.*
35. **Mossman, T.** 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods 65:55.*
36. **Rodbard, D., and G. R. Frazier.** 1975. Statistical analysis of radioligand assay data. *Methods Enzymol. 37:3.*
37. **Aggarwal, B. B., W. J. Henzel, B. Moffat, W. J. Kohr, and R. N. Harkins.** 1985. Primary structure of human lymphotoxin derived from 1788 lymphoblastoid cell line. *J. Biol. Chem. 260:2334.*
38. **Matsudaira, P.** 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J. Biol. Chem. 262:10035.*
39. **Tachachier, E., M. Robert-Guroff, R. C. Gallo, and M. S. Reitz.** 1989. Human T-lymphotrophic virus I-infected T cells constitutively express lymphotoxin in vitro. *Blood 73:194.*
40. **Wingfield, P., R. H. Pain, and S. Craig.** 1987. Tumor necrosis factor is a compact trimer. *FEBS Lett. 211:179.*
41. **Smith, R. A., and C. Baglioni.** 1987. The active form of tumor necrosis factor is a trimer. *J. Biol. Chem. 262:6951.*
42. **Aggarwal, B. B., B. Moffat, and R. N. Harkins.** 1984. Human lymphotoxin: production by a lymphoblastoid cell line, purification and initial characterization. *J. Biol Chem. 7259:686.*
43. **Kramer, S. M., M. E. Carver, and S. Apperson.** 1986. Comparison of TNF-α and TNF-β cytolytic biological activities in a serum free bioassay. *Lymphokine Res. 5:S139.*
44. **Lee, S. H., B. B. Aggarwal, E. Rinderknecht, F. Assisi, and H. Chiu.** 1984. The synergistic anti-proliferative effect of γ-interferon and human lymphotoxin. *J. Immunol. 133:1083.*
45. **Stone-Wolff, D. S., Y. K. Yip, H. C. Keller, J. Le, Henriksen-Destanfano, B. Y. Rubin, E. Rinderknecht, B. B. Aggarwal, and J. Vilcek.** 1984. Interrelationships of human interferon-γ with lymphotoxin and monocyte cytotoxin. *J. Exp. Med. 159:828.*
46. **Ruggiero, V., J. Travernier, W. Fiers, and C. Baglioni.** 1986. Induction of the synthesis of tumor necrosis factor receptors by interferon-γ. *J. Immunol. 136:2445.*
47. **Aggarwal, B., and T. E. Eessalu.** 1987. Induction of receptors for tumor necrosis factor-α by interferons is not a major mechanism for their synergistic cytotoxic response. *J. Biol. Chem. 262:10000.*
48. **Calvo, J. C, J. P. Radicella, and E. H. Charreau.** 1983. Measurement of specific radioactivities in labelled hormones by self-displacement analysis. *Biochem. J. 212:259.*
49. **Tsujimoto, M., Y. K. Yip, and J. Vilcek.** 1985. Tumor necrosis factor: specific binding and internalization in sensitive and resistant cells. *J. Immunol. 82:7626.*
50. **Cantor, C. R., and P. R. Schimmel.** 1980. The behavior of biological macromolecules. In *Biophysical Chemistry.* Part III. Freeman and Co., San Francisco, p. 860.
51. **Imamura, K., D. Spriggs, and D. Kufe.** 1987. Expression of tumor necrosis factor receptors on human monocytes and internalization of receptor bound ligand. *J. Immunol. 139:2989.*
52. **Ishikra, H., K. Hori, and A. Bloch.** 1989. Differential effects resulting from bimodal binding of recombinant human tumor necrosis factor to myeloid leukemia cells. *Blood 73:419.*
53. **Kull, F. C., S. Jacobs, and P. Cuatrecasas.** 1985. Cellular receptor for ^{125}I-labeled tumor necrosis factor: specific binding, affinity labeling and relationship to sensitivity. *Proc. Natl. Acad. Sci. USA 82:5756.*
54. **Creasey, A. A., R. Yamamoto, and C. R. Vitt.** 1987. A high molecular weight component of human tumor necrosis factor receptor is associated with cytotoxicity. *Proc. Natl. Acad. Sci. USA 84:3293.*
55. **Scheurich, P., E. Thoma, U. Uecer, and K. Pfizenmaier.** 1987. Immunoregulatory activity of recombinant human tumor necrosis factor (TNF)-α: induction of TNF receptors on human T cells and TNF-α mediated enhancement of T cell responses. *J. Immunol. 138:1786.*
56. **Chouaib, S., J. Bertoglio, J-Y. Blay, C. Marchiol-Fournigault, and D. Fradelizi.** 1988. Generation of lymphokine activated killer cells: synergy between tumor necrosis factor and interleukin-2. *Proc. Natl. Acad. Sci. USA 85:6875.*
57. **Ranges, G. E., M. P. Bombara, R. A. Aiyer, G. G. Rice, and M. A. Palladino.** 1989. Tumor necrosis factor-α as a proliferative signal for an IL-2 dependent T cell line: strict species specificity of action. *J. Immunol. 142:1203.*
58. **Plaetinck, G., W. Declercq, J. Travernier, M. Nabholz, and W. Fiers.** 1987. Recombinant tumor necrosis factor can induce interleukin-2 receptor expression and cytolytic activity in a rat × mouse T cell hybrid. *Eur. J. Immunol. 17:1835.*
59. **Fransen, L., M-R. Ruysschert, J. v.d. Heyden, and W. Fiers.** 1986. Recombinant tumor necrosis factor: species specificity for a variety of human and murine transformed cell lines. *Cell. Immunol. 100:260.*
60. **Stauber, G. B., R. A. Aiyer, and B. B. Aggarwal.** 1988. Human tumor necrosis factor-α receptor, purification by immunoaffinity chromatography and initial characterization. *J. Biol. Chem. 263:19098.*
61. **Kobayashi, Y., D. Miyaamotu, M. Asada, M. Obinata, and T. Osawa.** 1986. Cloning and expression of human lymphotoxin mRNA derived from a human T-cell hybridoma. *J. Biochem. 100:727.*
62. **Ruddle, N. H., and R. Homer.** 1988. The role of lymphotoxin in the inflammatory response. *Prog. Allergy 40:162.*
63. **Schaegger, H., and G. Jagow.** 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem. 166:368.*

Journal of Immunological Methods, 65 (1983) 55–63
Elsevier

JIM 02849

Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays

Tim Mosmann

DNAX Research Institute of Molecular and Cellular Biology, Inc., 1450 Page Mill Road, Palo Alto, CA 94304, U.S.A.

(Received 12 May 1983, accepted 20 June 1983)

A tetrazolium salt has been used to develop a quantitative colorimetric assay for mammalian cell survival and proliferation. The assay detects living, but not dead cells and the signal generated is dependent on the degree of activation of the cells. This method can therefore be used to measure cytotoxicity, proliferation or activation. The results can be read on a multiwell scanning spectrophotometer (ELISA reader) and show a high degree of precision. No washing steps are used in the assay. The main advantages of the colorimetric assay are its rapidity and precision, and the lack of any radioisotope. We have used the assay to measure proliferative lymphokines, mitogen stimulations and complement-mediated lysis.

Key words: *lymphokine assays – proliferation assays – colorimetric assay – tetrazolium – TCGF*

Introduction

Many biological assays require the measurement of surviving and/or proliferating mammalian cells. This can be achieved by several methods, e.g., counting cells that include/exclude a dye, measuring released ^{51}Cr-labeled protein after cell lysis, and measuring incorporation of radioactive nucleotides ([^{3}H]thymidine or [^{125}I]iododeoxyuridine) during cell proliferation. The radioactive method can be partially automated and can handle moderately large numbers of samples, but even with these methods, it is difficult to process thousands of assay points per day. In our current research we assay many samples of various lymphokines that induce cell proliferation, and so we required a rapid and quantitative assay capable of handling large numbers of samples.

Viable cells could be measured by using any of several staining methods, but we wished to avoid any washing steps that would increase processing time and sample variation. Multiwell scanning spectrophotometers (ELISA readers) can measure large numbers of samples with a high degree of precision, and so we investigated the possibility of using a color reaction as a measure of viable cell number. Ideally, a

0022-1759/83/$03.00

colorimetric assay for living cells should utilize a colorless substrate that is modified to a colored product by any living cell, but not by dead cells or tissue culture medium. Tetrazolium salts are attractive candidates for this purpose, since they measure the activity of various dehydrogenase enzymes (Slater et al., 1963). The tetrazolium ring is cleaved in active mitochondria, and so the reaction occurs only in living cells.

We have developed a rapid colorimetric assay, based on the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), that measures only living cells and can be read on a scanning multiwell spectrophotometer (ELISA reader). This assay is versatile and quantitative, and we consider it a significant advance over traditional techniques for several commonly used proliferation and cytotoxicity assays.

Materials and Methods

Cell lines

The EL_4G^- mouse lymphoma cell line was obtained from G. Carlson, and subclone EL_4.3 was selected for growth in 6-thioguanine. Another subline of EL_4, designated EL_4E2, was obtained from V. Paetkau. The EL_4E2 subline produces large quantities of interleukin 2 when stimulated with phorbol myristate acetate (Farrar et al., 1980). A continuous line of mouse T cells, A70 13/13, was derived in the author's laboratory at the University of Alberta. All cells were grown in RPMI 1640 supplemented with 50 μM 2-mercaptoethanol and 5–10% fetal bovine serum, in a 6% CO_2 atmosphere.

Colorimetric MTT (tetrazolium) assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma catalog no. M2128) was dissolved in PBS at 5 mg/ml and filtered to sterilize and remove a small amount of insoluble residue present in some batches of MTT. At the times indicated below, stock MTT solution (10 μl per 100 μl medium) was added to all wells of an assay, and plates were incubated at 37°C for 4 h. Acid-isopropanol (100 μl of 0.04 N HCl in isopropanol) was added to all wells and mixed thoroughly to dissolve the dark blue crystals. After a few minutes at room temperature to ensure that all crystals were dissolved, the plates were read on a Dynatech MR580 Microelisa reader, using a test wavelength of 570 nm, a reference wavelength of 630 nm, and a calibration setting of 1.99 (or 1.00 if the samples were strongly colored). Plates were normally read within 1 h of adding the isopropanol.

Interleukin 2 assay

Interleukin 2 (IL2) was derived from phorbol-myristate-acetate stimulated EL_4E2 cells (Farrar et al., 1980). The IL2-dependent T cell line A70 13/13 was used as an indicator cell. Doubling dilutions of IL2 were prepared in 96-well trays using growth medium as diluent. T cells (2000 per well) were then added, with a final volume of 0.1 ml per well. At 48 h, proliferation was measured by the MTT colorimetric assay.

Mitogen-induced proliferation of spleen cells

BALB/c mouse spleen cells were stimulated in 0.1 ml at 10^6 cells/well with varying concentrations of *Salmonella typhosa* lipopolysaccharide (LPS; Sigma) or concanavalin A (Con A; Calbiochem) and assayed at 3 days for proliferation using both colorimetric MTT and [^{3}H]thymidine incorporation assays. For the radioactive assay, 0.001 mCi [^{3}H]thymidine was added to each well, and after 4 h at 37°C the cells were harvested using a PHD cell harvester (Cambridge Instruments, Cambridge, MA).

Computer processing

Readings from the Dynatech MR580 Microelisa reader were transferred directly to an Apple II computer, using a program that saved the results to a diskette and printed the OD values in a 96-well format that matched the original plate. Additional programs were written to process the data stored on diskettes. We now have programs to plot results, calculate and plot means and standard deviations, identify wells above a chosen threshold, and calculate units of growth factor. These programs are available on request.

Results

In preliminary experiments, we tested several tetrazolium salts by incubation with cells for several hours. The most promising reagent was MTT, a pale yellow substrate that produced a dark blue formazan product when incubated with live cells. The MTT formazan reaction product was only partially soluble in the medium, and so an alcohol was used to dissolve the formazan and produce a homogeneous solution suitable for measurement of optical density. Initially, ethanol was used for this purpose, but some precipitation of serum proteins occassionally occurred in the acid-alcohol mixture. Several other organic solvents were tested, and isopropanol was found to be the most suitable solvent. Normal tissue culture medium has a variable color due to pH changes and the red form of phenol red interfered at the wavelength most suitable for blue MTT formazan measurement. To minimize this interference, we converted the phenol red to the fully acidic, yellow form at the end of the assay.

Our final procedure was to add 0.01 ml MTT (5 mg/ml in phosphate-buffered saline) to 0.1 ml cells in growth medium. After 4 h at 37°C for MTT cleavage, the formazan product was solubilized by the addition of 0.1 ml 0.04 N HCl in isopropanol. Optical density was measured on a Dynatech MR 580 plate reader, using a reference wavelength of 630 nm and a test wavelength of 570 nm.

EL_4.3 lymphoma cells were used to test the relationship between cell number and the amount of MTT formazan generated. The results in Fig. 1 show that the absorbance is directly proportional to the number of cells. This linearity extends over almost the entire range tested, from 50,000 to 200 cells/well. In addition, these results indicate that the assay is capable of detecting very small numbers of living cells (e.g., 200). The actual cells do not absorb significantly, even at a concentration of 1×10^6 cells/ml.

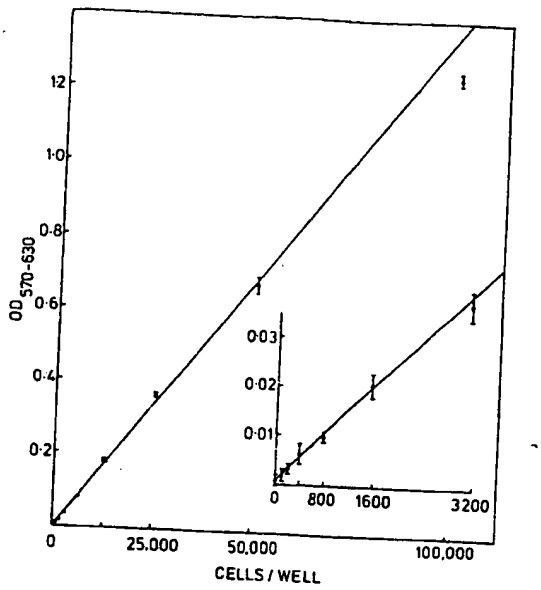

Fig. 1. Linearity of the MTT assay. EL_4.3 cells were plated out in doubling dilutions in 0.1 ml growth medium (RPMI 1640 + 10% fetal bovine serum) in 96-well flat-bottomed trays (Falcon), starting at 10^5 cells/well. MTT (0.01 ml of 5 mg/ml stock) was added immediately to all wells, and the plates were incubated at 37°C for 3 h, developed and measured. Each point shows the mean and standard deviation of 4 replicates. The straight line plotted is the best fit line calculated using all points from 100 to 50,000 cells/well.

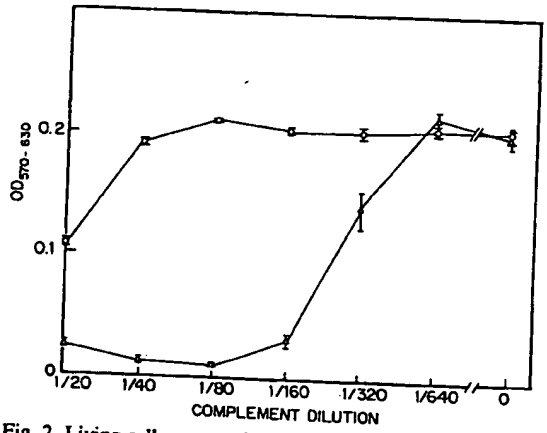

Fig. 2. Living cells are required for MTT cleavage. EL_4.3 cells were treated with anti-Thy1.2 (Cedarlane), and then treated cells and untreated controls were added to rabbit complement dilutions in a 96-well tray. After 30 min incubation at 37°C, MTT was added to all wells, and after another 4 h the plates were developed and read. Each point shows the mean and standard deviation of 4 replicates. △, anti-Thy1.2-treated; ○, untreated.

In many assays, dead cells will be present, and so it was important to determine if recently killed cells were positive or negative in the assay. Fig. 2 shows that only live cells actively cleave MTT, while dead cells are almost completely negative even immediately after complement-mediated lysis. These results suggested that living cells with active mitochondria are required to generate a strong signal, and raised the possibility that the amount of formazan generated per cell would depend on the level of energy metabolism in the cell. To test this, we measured formazan generation by metabolically inactive cells (red blood cells), resting cells (spleen cells) and activated cells (concanavalin A-stimulated lymphocytes). Fig. 3a shows that neither chicken nor sheep red blood cells cleave MTT to a significant extent, and neither red cell type interferes significantly in the assay, up to concentrations of 2×10^6 cells/ml. Fig. 3b shows that Con A-activated lymphocytes produce approximately 10 times as much formazan per cell as their normal counterparts.

A continuous line of interleukin 2 (IL2)-dependent T cells (A70 13/13), previously established in the author's laboratory at the University of Alberta, was used as

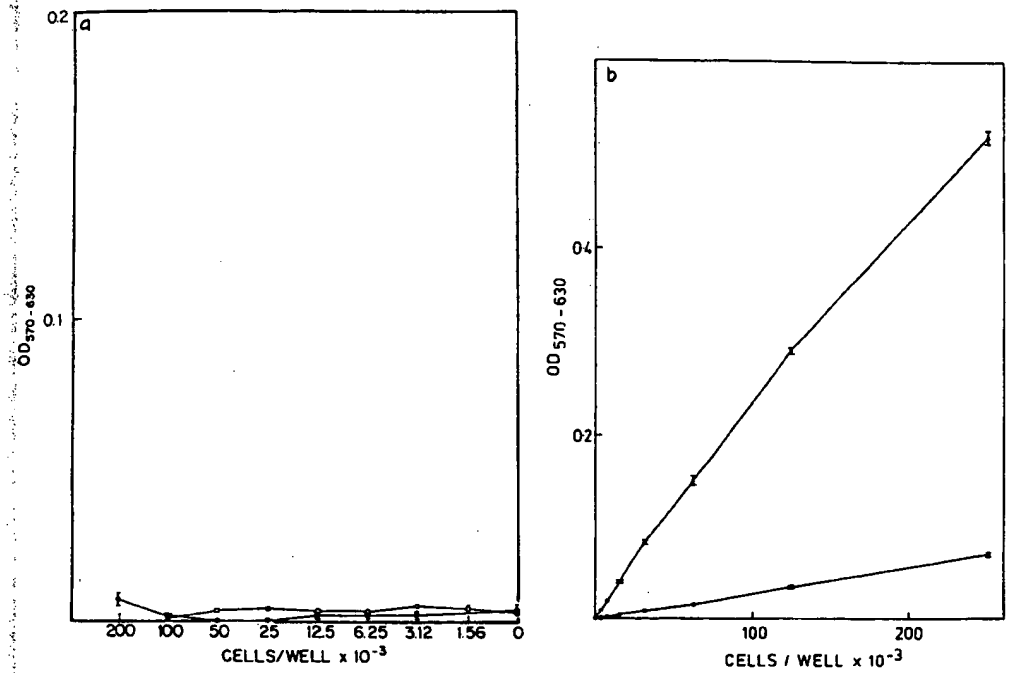

Fig. 3. MTT cleavage by erythrocytes and normal and activated lymphocytes. a: chicken and sheep erythrocytes were incubated in 0.1 ml medium with MTT for 3 h at 37°C. The plates were then developed and read. Means and standard deviations of 3 replicates per point are shown. ●, chicken erythrocytes; ○, sheep erythrocytes. b: mouse spleen cells were stimulated with 2 μg/ml concanavalin A for 48 h, and then the Con A-activated lymphocytes and normal lymphocytes were plated in doubling dilutions in 96-well flat-bottomed trays. MTT was added immediately, and after 2 h, the plates were developed and read. The means and standard deviations of 3 replicates per point are shown: ●, Con A-activated lymphocytes; ○, normal lymphocytes.

e plated out in doubling dilutions in 0.1 ml growth 96-well flat-bottomed trays (Falcon), starting at 10^5 added immediately to all wells, and the plates were Each point shows the mean and standard deviation it line calculated using all points from 100 to 50,000

$_4$.3 cells were treated with anti-Thy1.2 (Cedarlane), led to rabbit complement dilutions in a 96-well tray. to all wells, and after another 4 h the plates were l standard deviation of 4 replicates. △, anti-Thy1.2-

the target cell for an IL2 assay. This assay was used to test 4 parameters of th colorimetric reaction – the length of exposure of cells to IL2, the duration of MT treatment, the concentration of MTT used, and the number of test cells added to th assay.

Fig. 4 shows the effect of varying the time of incubation with MTT. The signa increased almost linearly from 1/2 to 2 h, but increased at a lesser rate from 2 to 4 h. In similar experiments, the concentration of MTT and cell number were optimized for the cell lines used in our studies (results not shown). The formazan generated was approximately proportional to the MTT concentration at low concentrations, and reached a plateau at about 0.45 mg/ml MTT. The formazan generated was also proportional to the number of cells at high IL2 concentrations, but the amount of factor required to produce 50% stimulation was increased at higher target cell concentrations. The assay could be read at 1, 2 or 3 days, but the apparent titer of the IL2 declined with increasing incubation time, probably due to depletion of the growth factor during cell growth. Our optimum values for these 4 parameters may need modifying for other assays, but in general, we have found that widely differing cell lines require only minimal changes.

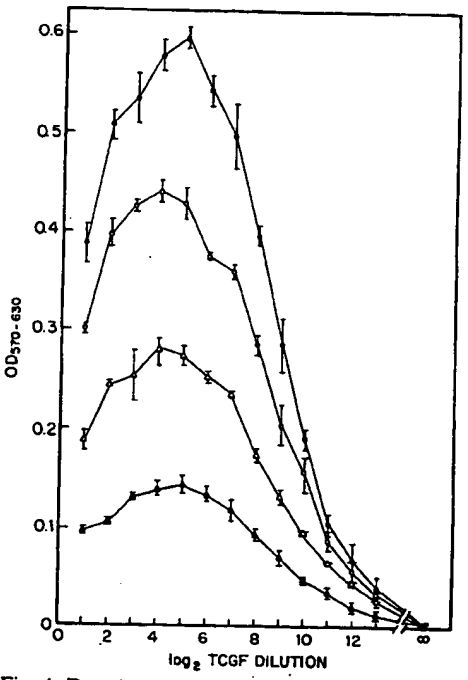

Fig. 4. Duration of MTT incubation. Interleukin 2 dilutions were assayed on A70 13/13 T cells. MTT was added at 44, 46, 47 and 47½ h, to sets of 3 rows each, and at 48 h, all wells were developed and read. Optical density readings were measured relative to control wells containing medium, cells and MTT but no growth factor. The means and standard deviations of 3 replicates per point are shown. ▲, 1/2 h; △, 1 h; O, 2 h; ●, 4 h.

ay was used to test 4 parameters of the
ıure of cells to IL2, the duration of MTT
and the number of test cells added to the

ime of incubation with MTT. The signal
but increased at a lesser rate from 2 to 4
ɔn of MTT and cell number were opti-
lies (results not shown). The formazan
to the MTT concentration at low con-
ɔout 0.45 mg/ml MTT. The formazan
ıber of cells at high IL2 concentrations,
duce 50% stimulation was increased at
could be read at 1, 2 or 3 days, but the
easing incubation time, probably due to
growth. Our optimum values for these 4
says, but in general, we have found that
nal changes.

ɔns were assayed on A70 13/13 T cells. MTT
ınd at 48 h, all wells were developed and read.
wells containing medium, cells and MTT but
replicates per point are shown. ▲, 1/2 h; △, 1

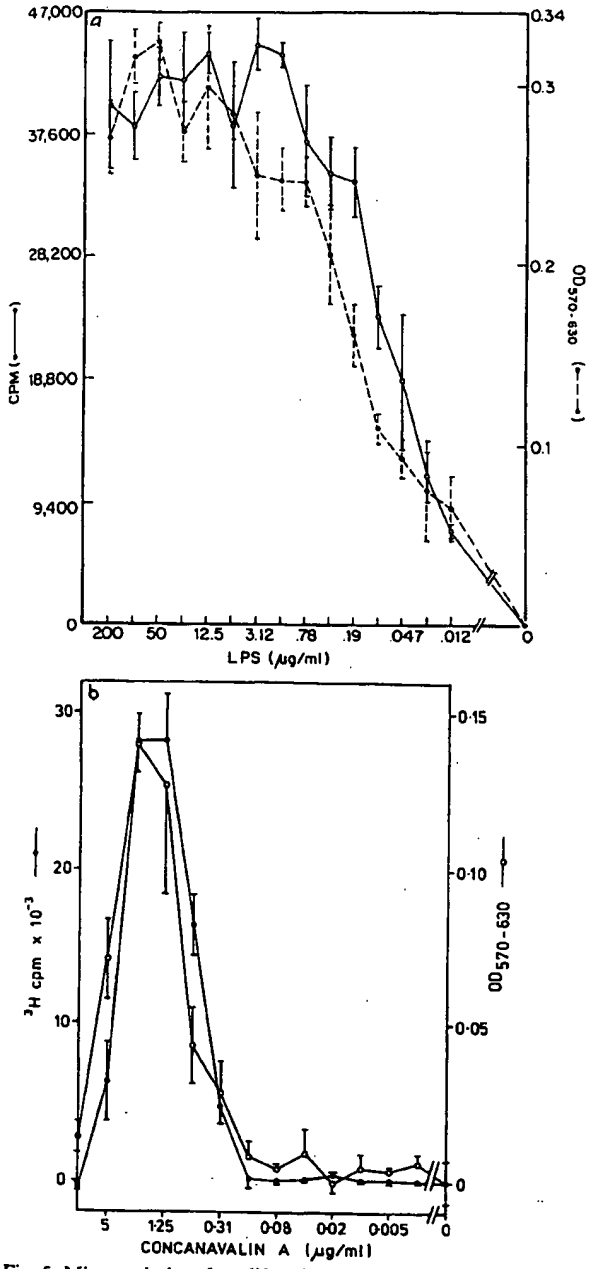

Fig. 5. Mitogen-induced proliferation of spleen cells. Spleen cells were stimulated for 3 days with varying concentrations of LPS and Con A and proliferation was measured using both colorimetric and radioactive assays. Results are shown as the means and standard deviations of 4 replicates per point. Background values, obtained from wells with cells but no mitogen, were subtracted from all points. a: LPS stimulation. b: Con A stimulation.

The results of the colorimetric assay with cloned cell lines were very encouraging, and so we explored the utility of the assay in more complex systems: the lymphocyte proliferative responses to the mitogens Con A and LPS. Since many cell types are present in the cell populations normally used for such proliferations, it was conceivable that certain cell types would generate abnormally large or small signals. Accordingly, we compared the colorimetric assay to a [^{3}H]thymidine incorporation assay for both Con A and LPS responses of normal mouse spleen cells.

Stimulation with both mitogens was measured effectively by both assays (Fig. 5). LPS stimulated cells over an extended concentration range, whereas the titration curve for Con A showed a narrow optimum, with little or no proliferation at high or low concentrations. The colorimetric and radioactive assays showed excellent agreement for Con A stimulations, and showed a small difference between the endpoint of LPS stimulations.

Activated macrophages produce more formazan product from nitroblue tetrazolium than do non-activated macrophages (Baehner et al., 1976), and so we measured MTT formazan production after LPS activation of a macrophage-like cell line, P388D1 (Lachman et al., 1977). No increase in MTT formazan production was seen after stimulation with a wide range of LPS concentrations, and P388D1 cells did not produce an unusual amount of MTT formazan (results not shown).

Discussion

The cleavage of MTT has several desirable properties for assaying cell survival and proliferation. MTT is cleaved by all living, metabolically active cells that we have tested, but not by dead cells or erythrocytes. The amount of formazan generated is directly proportional to the cell number over a wide range, using a homogeneous cell population. Activated cells produce more formazan than resting cells, which could allow the measurement of activation even in the absence of proliferation. These properties are all consistent with the cleavage of MTT only by active mitochondria.

The main advantage of the colorimetric assay is the speed with which samples can be processed. The substrate does not interfere with measurement of the product, and we have found conditions in which components of the medium do not interfere. This allows the assay to be read with no removal or washing steps, which increases the speed of the assay and helps to minimize variability between samples. The final stages of the assay (adding the MTT, reading the plate and printing the data) take much less time than setting up the assay (mixing cells and growth factor dilutions). The assay can be read a few minutes after the addition of acid-isopropanol, and the color is stable for a few hours at room temperature. The results are also apparent visually, which is very useful if rapid qualitative results are required.

The colorimetric assay measures the number and activity of living cells at the end of the assay, whereas [^{3}H]thymidine incorporation measures the number of cells synthesizing DNA during the last few hours of the assay. So the colorimetric assay correlates well with visual examination of the cells at the end of the assay (Kapplei

n cloned cell lines were very encouraging,
n more complex systems: the lymphocyte
n A and LPS. Since many cell types are
d for such proliferations, it was conceiva-
normally large or small signals. Accord-
to a [^{3}H]thymidine incorporation assay
nal mouse spleen cells.
sured effectively by both assays (Fig. 5).
ncentration range, whereas the titration
, with little or no proliferation at high or
dioactive assays showed excellent agree-
a small difference between the endpoint

ormazan product from nitroblue tetra-
es (Baehner et al., 1976), and so we
-PS activation of a macrophage-like cell
rease in MTT formazan production was
LPS concentrations, and P388D1 cells
formazan (results not shown).

le properties for assaying cell survival
ing, metabolically active cells that we
ythrocytes. The amount of formazan
ll number over a wide range, using a
s produce more formazan than resting
of activation even in the absence of
ent with the cleavage of MTT only by

ay is the speed with which samples can
with measurement of the product, and
ts of the medium do not interfere. This
or washing steps, which increases the
ariability between samples. The final
; the plate and printing the data) take
ing cells and growth factor dilutions).
addition of acid-isopropanol, and the
erature. The results are also apparent
ve results are required.
r and activity of living cells at the end
ration measures the number of cells
f the assay. So the colorimetric assay
cells at the end of the assay (Kappler

et al., 1981) but these 2 assays can potentially differ from radioactive nucleotide incorporation methods. This should be kept in mind for specific applications, e.g., distinguishing between death, survival and proliferation. In practice, we have not seen large differences between the colorimetric assay, radioisotope assay or visual inspection of the wells.

The only additional reagents used in the assay are MTT, isopropanol, and HCl. No radioisotopes are used, and no scintillation counter or gamma-counter is needed. This advantage is partially offset by the requirement for a plate reader, but the high scanning rate of typical machines (e.g., $1\frac{1}{2}$ min per 96 wells) allows a single plate reader to handle very large numbers of samples.

The colorimetric assay shares with the radioisotope assays the advantages of precise quantitation and compatibility with computer analysis programs. Since the colorimetric assay is so rapid, large amounts of data can be generated, and some form of computer processing is very desirable. We have set up programs for calculating means and standard deviations, plotting curves, and calculating units of growth factor in the original sample (using a linear interpolation to calculate the exact dilution at which stimulation is a preset value, e.g., 25% of the maximum plateau stimulation). These programs are written for an Apple II computer, and are available on request.

The reduction of MTT to a formazan product appears to be carried out by all the cell types we have examined. These include mitogen stimulated T and B cells, myeloma, T lymphoma and macrophage-like tumor cell lines, as well as various IL2-dependent T cell lines. This suggests that the colorimetric MTT assay may have very wide applicability for measuring survival and/or proliferation of various cells and can potentially be applied to any assay in which living cells must be distinguished from dead cells or a lack of cells. The results in Fig. 2 show that dead cells are unable to cleave MTT within 30 min of complement-mediated lysis. This indicates that the assay also has potential value for quantitative and rapid measurement of cell death, e.g., in HLA typing. The MTT assay may also be applicable to the assay of cytotoxic T lymphocytes, although the signal generated by the CTL population could mask the signal from the target population at high effector : target ratios.

Acknowledgements

I thank M. Kerkhove, D. Lancaster and J. Wrobel for excellent technical assistance, and J. Abrams and M. Tomasello for help with the computer programs.

References

Baehner, R.L., L.A. Boxer and J. Davis, 1976, Blood 48, 309.
Farrar, J.J., J.F. Farrar, P.L. Simon, M.L. Hifliker, B.M. Stadler and W.L. Farrar, 1980, J. Immunol. 125, 2555.
Kappler, J.W., B. Skidmore, J. White and P. Marrack, 1981, J. Exp. Med. 153, 1198.
Lachman, L.B., M.P. Hacker, G.T. Blyden and R.E. Handschumaker, 1977, Cell. Immunol. 34, 416.
Slater, T.F., B. Sawyer and U.D. Strauli, 1963, Biochim. Biophys. Acta 77, 383.

Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 6197–6201, July 1992
Medical Sciences

Selective stimulation of prostatic carcinoma cell proliferation by transferrin

(prostate cancer/tumor metastasis/growth factors)

MARCELA CHACKAL ROSSI* AND BRUCE R. ZETTER†‡

*Department of Biological Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139; and †Department of Surgery and Department of Cellular and Molecular Physiology, Children's Hospital and Harvard Medical School, Boston, MA 02115

Communicated by Judah Folkman, March 20, 1992

ABSTRACT **Aggressive prostatic carcinomas most frequently metastasize to the skeletal system. We have previously shown that cultured human prostatic carcinoma cells are highly responsive to growth factors found in human bone marrow. To identify the factor(s) responsible for the increased prostatic carcinoma cell proliferation, we fractionated crude bone marrow preparations by using hydroxylapatite HPLC. The major activity peak contained two high molecular weight bands (M_r = 80,000 and 69,000) that cross-reacted with antibodies to human transferrin and serum albumin, respectively. Bone marrow transferrin, purified to apparent homogeneity by using DEAE-Affi-Gel Blue chromatography, anti-transferrin affinity chromatography, and hydroxylapatite HPLC, markedly stimulated prostatic carcinoma cell proliferation, whereas human serum albumin showed no significant growth factor activity. Marrow preparations, depleted of transferrin by passage over an anti-transferrin affinity column, lost >90% of their proliferative activity. In contrast to the response observed with the prostatic carcinoma cell lines, a variety of human malignant cell lines, derived from other primary sites and metastatic to sites other than bone marrow, showed a reduced response to purified marrow-derived transferrin. These results suggest that rapid growth of human prostatic carcinoma metastases in spinal bone may result from a combination of conditions that include (*i*) drainage of prostatic carcinoma cells into the paravertebral circulation, (*ii*) high concentrations of available transferrin in bone marrow, and (*iii*) increased sensitivity of prostatic carcinoma cells to the mitogenic activity of transferrin.**

Prostatic carcinoma and lung cancer represent the most commonly diagnosed malignancies in older American males and are the predominant causes of male cancer deaths (1, 2). Metastatic prostate carcinoma shows a site-specific localization, with the majority of metastases occurring in bone, especially in the vertebrae, where metastatic growth is rapid and virulent (3, 4). In contrast, growth of primary prostatic carcinoma is remarkably slow, often extending over decades.

The preferential colonization of bone marrow by prostatic carcinoma has been attributed to the passage of cells from the prostate to the spine via specialized paravertebral blood vessels first described by Batson (5, 6). The existence of these channels may contribute to the preferential vertebral seeding of prostatic tumor cells but does not address the discrepancy between the apparent growth rates for secondary prostatic tumors in the bone versus primary tumors in the prostate. We have previously reported that bone marrow contains factors that are highly stimulatory for two human prostatic carcinoma cell lines derived from metastatic lesions (7). A variety of known hematopoietic and nonhematopoietic growth factors were subsequently tested for their ability to stimulate prostatic carcinoma cell growth, but none had substantial activity (7).

In the present study, we describe the purification of a mitogenic factor for human prostatic carcinoma cells from human bone marrow. Our results reveal that the purified activity resides in transferrin (Tf), an iron-transporting molecule found in high concentration in bone marrow. In addition, prostatic carcinoma cells show an increased responsiveness to the growth-promoting activity of Tf relative to several other metastatic tumor cell types. This combination of high responsiveness of prostatic carcinoma cells to Tf along with a high concentration of Tf in bone marrow may partially explain the accelerated growth observed for prostatic carcinoma metastases localized to bony sites.

MATERIALS AND METHODS

Cells. All human metastatic cell lines were obtained from the American Type Culture Collection. Details of the isolation and characterization of the two prostatic carcinoma cell lines, PC-3 and DU 145, have been published (8, 9). Culture conditions for all cell lines were as described (7). Characterized fetal bovine serum (FBS; HyClone) containing 1.33 mg of Tf per ml was heat-treated for 30 min at 56°C before use.

Preparation of Crude Marrow-Derived Growth Factor. Human spinal bone marrow discarded from bone marrow transplant procedures was used as starting material for these studies. Crude aspirates of bone marrow were washed with sterile RPMI 1640 (GIBCO), and the insoluble material was centrifuged at 700 × *g* for 5 min. The supernatant was discarded, and the pellet was resuspended in 5 volumes of sterile culture medium containing penicillin (100 units/ml) and streptomycin (100 μg/ml; Irvine Scientific) and incubated in 5% CO_2 for 24 hr at 37°C (7). The resulting preparation was clarified by centrifugation at 800 × *g* for 5 min to remove cells. The total protein concentration in the starting material ranged from 1 to 3 mg/ml as determined using the Bio-Rad protein assay. For comparison with other tissues, 4 g of the marrow samples or frozen kidney, liver, skin, or lung, obtained from the National Disease Research Interchange (Philadelphia), was minced, rinsed with water, centrifuged, resuspended, and extracted in 20 ml of water for 3 hr. Particulate matter was removed by centrifugation, and the supernatant was collected, analyzed for protein content using the Pierce BCA protein assay, and applied directly to SDS/PAGE gels.

Cell Proliferation Assays. Cells were seeded at 2000 cells per well in 96-well tissue culture dishes (Costar) containing RPMI 1640 medium with 0.25–0.5% FBS, glutamine, peni-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: Tf, transferrin; HSA, human serum albumin; FBS, fetal bovine serum.

‡To whom reprint requests should be addressed at: Children's Hospital, Harvard Medical School, 300 Longwood Avenue, Boston, MA 02115.

cillin, and streptomycin and were allowed to plate overnight. After 18 hr, test preparations including crude bone marrow, column fractions, marrow-derived Tf, and human plasma Tf (Calbiochem) were added to the medium in each well (day 1). The cells were incubated for 3 additional days without change of medium and terminated on day 4. Cell number was estimated by using a colorimetric assay (10). Twenty microliters of the reagent 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) at 5 mg/ml in Dulbecco's phosphate-buffered saline (PBS) was added to each well and incubated for 4 hr at 37°C, and the cells were lysed with 100 μl of 10% SDS/0.1 M HCl to solubilize the blue formazan crystal product (11). The plates were incubated at 37°C overnight, and the absorbance at 600 nm was detected by using a Dynatech automated microplate ELISA reader. For each assay, absorbance values were normalized to a standard curve generated with known numbers of PC-3 cells.

Cell proliferation assays involving other malignant cell types were performed in 24-well tissue culture dishes (Costar). Cells were seeded at initial densities of 10^4 cells per well in RPMI medium containing 0.25% FBS and allowed to plate overnight. The next day (day 1), various doses of purified marrow-derived Tf were added to the wells. The experiments were terminated on day 4 by trypsinization, and the cell number was determined by using a Zf particle counter (Coulter).

Biochemical and Immunological Procedures. For gel electrophoresis (12), samples were heat-treated for 3 min at 95°C in sample buffer with 1.0% 2-mercaptoethanol (Bio-Rad) and applied to the gel. Gels were silver stained (13) or proteins were transferred onto nitrocellulose paper (14) by using a PolyBlot semidry transfer apparatus (American Bionetics, Hayward, CA). Blots were probed with either goat anti-human Tf (1:250 dilution; Calbiochem) or rabbit anti-human serum albumin (HSA) IgG (1:750 dilution; Boehringer Mannheim) and resolved with protein A-conjugated alkaline phosphatase (1:1000 dilution; Boehringer Mannheim). The bands were visualized by using a Bio-Rad alkaline phosphatase substrate kit.

DEAE-Affi-Gel Blue Chromatography. DEAE-Affi-Gel Blue (Bio-Rad Labs; ref. 15) was washed with PBS and packed into a 1 × 17 cm column to give a total bed volume of 14 ml. Crude marrow-derived material (20 mg in 10 ml) was passed over the column at a flow rate of 0.5 ml/min. The column was washed with 5 volumes of PBS, and unbound material was collected and concentrated 10-fold by using a Centriprep-10 concentrator (Amicon).

Anti-Tf-Sepharose Chromatography. Rabbit anti-human Tf IgG (4 mg/ml) was coupled to CNBr-activated Sepharose 4B (Pharmacia LKB Biotechnology) according to the manufacturer's instructions. Concentrated material from the DEAE-Affi-Gel Blue chromatography step was applied to a 1 × 8 cm anti-Tf column (bed volume, 7 ml). The column was equilibrated with 5 volumes of 0.1 M phosphate/0.2 M citrate/0.25 M NaCl, pH 7.0 (16). Bound proteins were eluted with 5 column volumes of the same buffer at pH 2.8 at a flow rate of 0.5 ml/min. Unbound and bound fractions were collected, pooled, and concentrated 10-fold in Centricon-10 microconcentrators (Amicon). For further purification, bound fractions were applied to hydroxylapatite HPLC columns as described below.

Hydroxylapatite HPLC. Crude marrow-derived material (200 μg in 2 ml of 1 mM phosphate at pH 7.0) was applied to a 100 × 7.8 mm Bio-Gel hydroxylapatite HPLC column (Bio-Rad) and eluted with a gradient of 1–200 mM Na_3PO_4. Fractions (1 ml) were collected, passed through a 0.2-μm filter (Gelman), and assayed for biological activity.

RESULTS

Hydroxylapatite HPLC Fractionation of Crude Bone Marrow-Derived Growth Factors. Two hundred micrograms of total protein from crude bone marrow was loaded onto a hydroxylapatite HPLC column. Bound proteins were eluted with a continuous sodium phosphate gradient from 1 to 200 mM. The elution profile and distribution of mitogenic activity are shown in Fig. 1. Most of the growth stimulatory activity was detected as a major peak eluting at 120 mM Na_3PO_4. A smaller activity peak was observed immediately after the void volume. The proliferative activity is plotted as the percent increase in cell number above background after 4 days, where background levels represent cells grown in 0.5% FBS. Crude marrow preparations, used as a positive control, showed a 125% increase in cell growth after 4 days. Fractions were analyzed by silver stained SDS/PAGE as shown in Fig. 2*A*. Two major bands were observed in the active fractions, the larger at M_r 80,000 and a smaller, more abundant band at M_r 69,000.

Because two major components of bone marrow are Tf (M_r, 77,000–80,000) and HSA (M_r, 69,000), we analyzed the hydroxylapatite fractions for cross-reactivity with antibodies to these two molecules (Fig. 2*B*). The immunostaining confirmed the presence of both Tf and HSA in the material eluted from hydroxylapatite. As revealed in the elution pattern shown in Fig. 1, the mitogenic activity was found in fractions 34–43, which contained both Tf and HSA. In contrast, fractions, such as no. 47, which contained only HSA, displayed no significant mitogenic activity. In light of these findings, we attempted to purify Tf from human bone marrow to determine its ability to stimulate prostatic carcinoma cell growth. The abundance of Tf in bone marrow relative to several other human tissues, as reported by De Jong *et al.* (17), was confirmed in our preparations by immunoblotting with antibodies to human Tf (Fig. 3). High levels of Tf in the lung, as shown here, have recently been reported by Cavanaugh and Nicolson (18).

Purification of Bone Marrow-Derived Tf. To remove albumin from crude marrow preparations, 2 ml of crude marrow (2 mg/ml) was loaded onto a DEAE-Affi-Gel Blue column. The column was washed with 5 volumes of PBS at pH 7.0, and the unbound material was collected and concentrated 10-fold. This step effectively reduces the concentration of albumin, as shown in the "Affigel" lane of the polyacrylam-

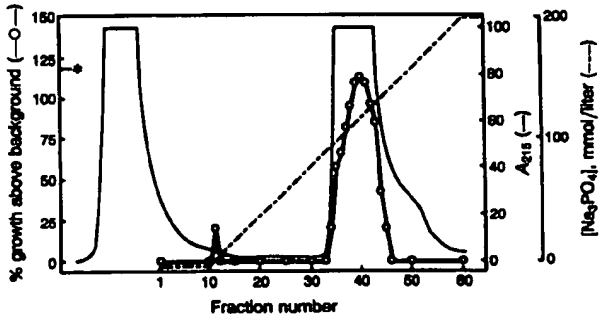

FIG. 1. Hydroxylapatite HPLC of crude marrow-derived growth factors. Crude marrow (200 μg) was diluted in 2 ml of 1 mM Na_3PO_4 at pH 7.0 and injected onto an HPLC hydroxylapatite column. The elution profile was monitored by measurement of absorbance at 215 nm. Each fraction (20 μl) was tested for its ability to stimulate proliferation of PC-3 prostatic carcinoma cells. Data is expressed as the mean (n = 3) percent increase in cell number above background, where background represents growth of cells in medium supplemented with 0.5% FBS plus 20 μl of buffer from equivalent fractions of a gradient run in the absence of bone marrow. Crude marrow-conditioned medium (100 μg/ml) was used as a positive control.

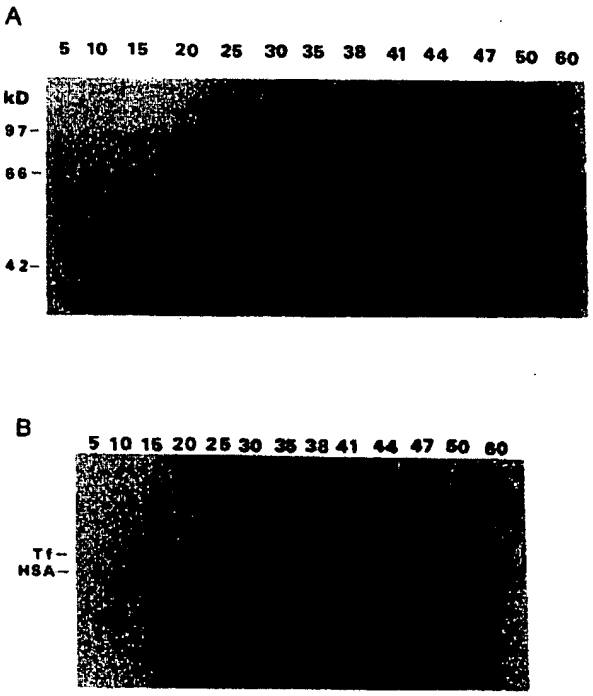

FIG. 2. SDS/PAGE gel analysis of hydroxylapatite fractions of crude bone marrow growth factors. Fractions eluted from hydroxylapatite HPLC were applied to a SDS/12.5% polyacrylamide gel under reducing conditions. The gel was subsequently silver stained (*A*), or the proteins were transferred onto nitrocellulose and tested for immunoreactivity to antibodies prepared against human plasma Tf or HSA (*B*). The fraction numbers are indicated along the top of the gels.

ide gel shown in Fig. 4*A*. The presence of HSA and Tf in these samples was monitored by immunoblotting with antibodies to these two proteins, as shown in Fig. 4*B*. Control experiments showed no cross-reactivity between Tf and albumin. This material was subsequently loaded onto an anti-Tf column and washed with 5 column volumes of 0.1 M phosphate/0.2 M citrate/0.25 M NaCl, pH 7.0; and the bound material was eluted with the same buffer at pH 2.8. This procedure results in material containing predominantly Tf with a small amount of residual proteins, as shown in the "Anti-Tf" lane in Fig. 4*A*. The bound fractions from the anti-Tf column were pooled, concentrated 10-fold, rediluted 2-fold in phosphate buffer at pH 7.0, and applied to a hydroxylapatite HPLC column. The activity corresponded to one sharp peak that eluted with 100 mM phosphate (data not shown). The gel profile of the fractions is shown in the "HAP" lane in Fig. 4*A*.

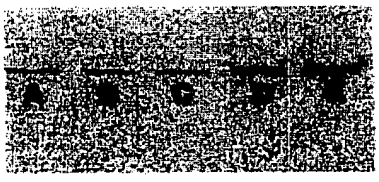

FIG. 3. Immunoblot analysis of human organ extracts with antisera to human Tf. Extracts were prepared as described in *Materials and Methods*, and 3 μg was loaded onto each lane of a SDS/4–20% polyacrylamide gradient gel containing 1.0% 2-mercaptoethanol. The proteins were transferred to nitrocellulose, and the blot was probed with an antibody to human Tf. Lanes: A, kidney; B, liver; C, skin; D, bone marrow; E, lung.

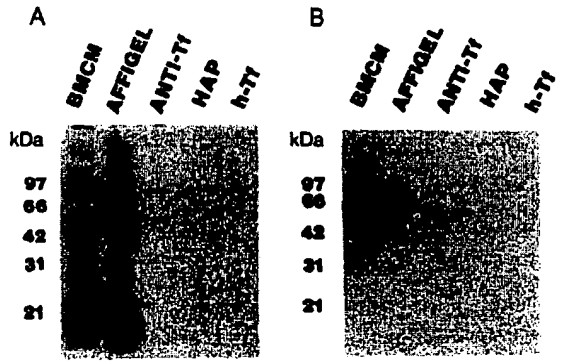

FIG. 4. Gel analysis of active fractions from purification scheme for marrow-derived Tf. Aliquots from the sequential purification steps were analyzed by silver staining (*A*) and immunoblotting of SDS/polyacrylamide gels with goat anti-human Tf (*B*). Lane BMCM, 1.5 μg of crude marrow; lane Affigel, 1.5 μg of material eluted from DEAE-Affi-Gel Blue; lane anti-Tf, 0.3 μg of material that bound to an anti-Tf affinity column; lane HAP, 0.15 μg of pooled active fractions 36 and 37 from hydroxylapatite HPLC; lane h-Tf, standard of 0.3 μg of purified human plasma Tf.

A single silver-stained band migrating with M_r 80,000 was obtained after the hydroxylapatite step.

Biological Activity of Bone Marrow-Derived Tf. The purified marrow-derived Tf was tested for its ability to stimulate PC-3 cell growth. As shown in Fig. 5, the purified Tf is a potent stimulator of PC-3 cell growth, with half maximal activity at ≈1.5 μg/ml. In contrast, HSA had little mitogenic activity for the PC-3 cells.

Inhibition of Marrow-Derived Growth Factor Activity by Antibodies to the Human Tf Receptor. Antibody 42/6, kindly provided by I. Trowbridge, prepared against the external domain of the human Tf receptor (19), has been shown to block Tf binding to ovarian carcinoma cells and to retard growth in a variety of tumor cell types (20). When this antibody was tested for its effect on the proliferation of prostatic carcinoma cells, the extent of inhibition for cells stimulated by crude marrow preparations was equivalent to that for cells stimulated by purified, iron-saturated human

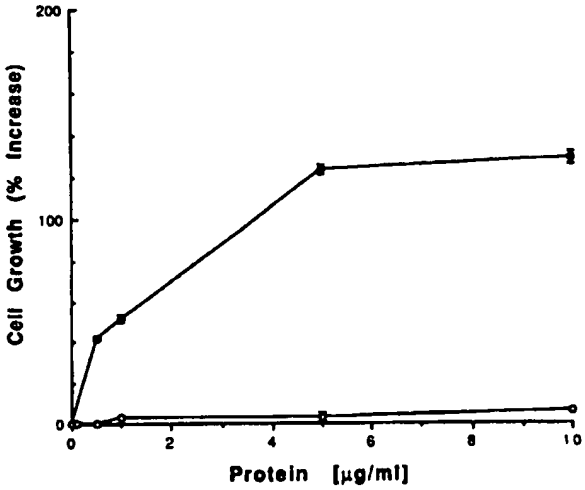

FIG. 5. Growth response of PC-3 to purified human bone marrow-derived Tf. Cells were seeded at 2000 cells per well in a 96-well tissue culture plate in medium containing 0.5% FBS and the indicated concentrations of purified bone marrow-derived Tf (●) or HSA (○).

plasma Tf. These results, as shown in Table 1, are consistent with a requirement for Tf to support growth of prostatic carcinoma cells.

Tf Depletion Results in Reduced Growth Factor Activity in Crude Marrow Preparations. Crude marrow preparations were depleted of Tf by two successive passes over an anti-Tf affinity column. The depleted fractions were tested for their ability to stimulate PC-3 cell growth. As shown in Table 2, Tf depletion effectively removed nearly all of the growth-stimulatory activity from crude marrow-derived material. After elution with a low pH buffer (pH 2.8) and subsequent neutralization, this material retained substantial growth factor activity. SDS gel staining and Western blotting (not shown) confirm the presence of Tf as the principal component of the eluant.

Response of Other Metastatic Cell Lines to Tf. The effect of marrow-derived Tf on the growth of a variety of metastatic tumor cells is shown in Fig. 6. No substantial response was seen with Caki-1 (human kidney carcinoma, metastatic to skin), Hs746t (human stomach carcinoma, metastatic to leg), Sk-Mel 2 (human malignant melanoma, metastatic to skin of thigh), MCF-7 (human breast carcinoma, pleural effusion), and Calu-1 (human lung epidermoid carcinoma, metastatic to pleura) cells. In contrast, PC-3 and Du 145, metastatic human prostatic carcinoma cell lines, were highly responsive to the mitogenic activity of marrow-derived Tf.

DISCUSSION

The preferential colonization of particular tissues by certain metastatic tumor cells is mediated by a combination of highly specific cellular interactions including site-specific cell adhesion, cell migration, and cell growth (21). In the case of prostatic carcinoma, a leading cause of cancer mortality for which no curative therapy currently exists, metastasis to bone is associated with poor survival (2–4). Preferential prostatic carcinoma metastasis to bone is mediated, in part, by preferential dissemination of cells to spinal bone via the paravertebral vessels (5, 6). Preferential growth of prostatic carcinoma in a variety of bone sites including the spine, long bones, and skull may be related to the finding that bone marrow contains growth factors that stimulate prostatic carcinoma cell growth (7). In the present report, we have demonstrated that Tf, purified from human bone marrow, is highly mitogenic for human prostatic carcinoma cells but less stimulatory for a variety of other metastatic cell lines.

Tf is a well-studied glycoprotein of M_r 77,000–80,000 that participates in iron transport in most tissues (22). Most Tf is produced in the liver where it is secreted into the bloodstream and transported to all tissues. The tissue distribution of Tf is not, however, homogeneous. Endogenous Tf is produced by a variety of nonhepatic cells including testicular sertoli cells, brain capillary endothelial cells, ependymal cells in the choroid plexus, and brain oligodendroglial cells (23). Tf is also sequestered in certain organs that are sites of iron absorption, utilization, and storage. Bone marrow is a principal site of iron utilization due to the high iron requirements of developing erythroid cells and rapidly dividing hematopoietic progenitors. This is reflected in the high concentrations of Tf found in normal red bone marrow (16). Prostate, in contrast, is normally low in Tf, but levels are elevated in hyperplastic or neoplastic prostatic tissue (24).

In vitro studies have demonstrated that cells cultured in defined serum-free medium require Tf in addition to other hormones and growth factors for cellular proliferation (25). In most cases, Tf-mediated iron transport is permissive for cell growth in the presence of additional growth factors. Tf receptor levels increase on proliferating cells, implying a requirement for increased iron transport associated with cell proliferation. Tf, by itself, is generally not mitogenic, but there are cells in which Tf alone can initiate passage through

Table 1. Inhibition of marrow-derived growth factor activity by antibody 42/6 to the human Tf receptor

Antibody concentration, μg/ml	% inhibition	
	Tf, 1 μg/ml	Crude marrow, 25 μg/ml
0	—	—
1	32	32
10	37	47
100	40	53

PC-3 cells were plated at a density of 2000 cells per well in a 96-well tissue culture plate in culture medium containing 0.25% FBS. After 18 hr, the indicated doses of plasma Tf, crude bone marrow preparation, and anti-Tf receptor were added to each well. The experiment was terminated on day 4. Data is expressed as the percent inhibition of cell growth relative to control cultures grown in the absence of the anti-Tf antibody.

Table 2. Crude human bone marrow preparation depleted of Tf by anti-Tf chromatography

Test material	% growth stimulation
Crude marrow	75 ± 4
Tf-depleted fractions	6.3 ± 2.6
Eluted Tf	70 ± 3.8

Crude bone marrow was depleted of Tf by two successive passages over a column containing anti-human Tf antibody conjugated to Sepharose 4B. Marrow-derived Tf was eluted from the column with phosphate/citrate buffer, pH 2.8, and subsequently neutralized. PC-3 cells were plated at 2000 cells per well in a 96-well tissue culture plate in medium containing 0.5% FBS. After 18 hr, saturating amounts of crude marrow (150 μg/ml) and column fractions (70 μg/ml) were added to the culture wells. The experiment was terminated on day 4. Data is expressed as the percent increase in cell number relative to control cells incubated in 0.5% serum alone.

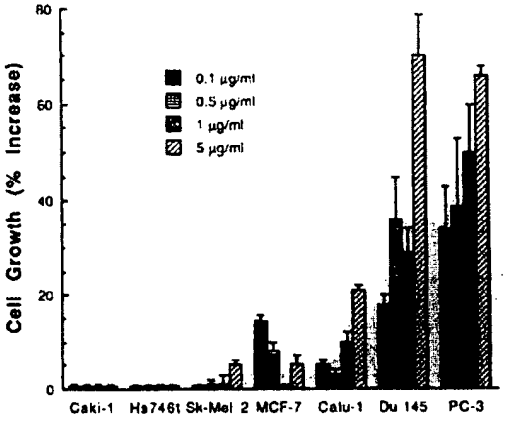

FIG. 6. Growth of human tumor cell lines in response to marrow-derived Tf. Cells were plated overnight at a density of 10^4 cells per 16-mm tissue culture well in 1% FBS. The next day, the wells were washed twice with sterile PBS, and fresh RPMI medium with 0.1% FBS was added to each well in addition to the indicated doses of purified marrow-derived Tf. After 4 days, the cells in each well were trypsinized and counted. Data are expressed as the mean ± SD (n = 3) for the percent increase above background. The cell lines tested include Caki-1 (human kidney carcinoma, metastatic to skin), Hs746t (human stomach carcinoma, metastatic to leg), Sk-Mel 2 (human malignant melanoma, metastatic to skin of thigh), MCF-7 (human breast carcinoma, pleural effusion), Calu-1 (human lung epidermoid carcinoma, metastatic to pleura), PC-3 (prostatic carcinoma, metastatic to bone), and DU 145 (prostatic carcinoma, metastatic to bone and brain).

the cell cycle. This is true for some tumor cells and particularly for leukemic cells (26–28).

The mechanisms underlying Tf-stimulated cell proliferation have been most extensively studied in hematopoietic cells (29). Under normal conditions, iron uptake requires the presence of specific cell surface receptors for Tf, and Tf receptors are expressed at high densities on the surface of rapidly proliferating cells. Terminally differentiated cells have few or no specific binding sites for Tf (30). Malignant cells generally also have more Tf receptors than cells in benign lesions (31).

The prostatic carcinoma cell lines PC-3 and DU 145 have been reported to have abundant Tf receptors on their cell surface (32). We now report that these cells proliferate in response to marrow-derived Tf, whereas a variety of human tumor cells metastatic to sites other than bone marrow were substantially less responsive to the mitogenic activity of the same Tf preparations. Previous results from our laboratory have demonstrated that these nonprostatic tumor cell lines were also relatively unresponsive to crude preparations of bone marrow-derived growth factors. We do not mean to imply, however, that *only* prostatic carcinoma cells will respond to Tf since some other tumor cells have been shown previously to proliferate in response to Tf (26–28). Certain lung-colonizing tumors have also been shown to proliferate in response to a Tf-like growth factor purified from lung tissue (33). Metastatic tumor cells that metastasize to other Tf-rich sites such as brain and testis might also be predicted to respond to Tf as a growth factor. Our results demonstrate that the growth response to Tf is not a universal feature of all metastatic tumor cells and may therefore have implications for the colonization of Tf-rich tissues by distinct populations of metastatic tumor cells.

No significant difference was observed between plasma Tf and marrow-derived Tf or between iron-saturated Tf and apotransferrin in our studies (data not shown), although the presence of iron in the 0.5% FBS used in these assays suggests that the apotransferrin would rapidly become iron-loaded under these conditions. Depletion of Tf from crude marrow preparations resulted in a >90% decrease in mitogenic activity, suggesting that Tf is required for growth stimulation of prostatic carcinoma cells.

Our current results present one possible explanation for the predominance of prostatic carcinoma metastases in vertebral bone. Passage of metastatic tumor cells from the prostate to the spine is promoted by the presence of specialized paravertebral vascular channels (5, 6). Once lodged in the vertebral marrow cavity, prostatic carcinoma cells may proliferate rapidly under the influence of high concentrations of growth factors such as Tf as well as other, as yet unidentified factors. Metastases growing in spinal bone may then shed additional tumor cells into the venous circulation, giving rise to tertiary metastases in other Tf-rich sites including lung, brain, and other bones. We hypothesize, therefore, that the preferential seeding of prostatic carcinoma to vertebral bone along with the selectively rich growth conditions in the marrow environment may account for the preferential colonization of the vertebrae by metastatic prostatic carcinoma cells. Prostatic carcinoma may therefore be a good candidate for treatment with antibodies to Tf or the Tf receptor or with the Tf receptor conjugated to bacterial toxins (34).

We are grateful to Drs. John Essigmann, Vernon Ingram, Michael Klagsbrun, Bing Lim, and Robert Langer for helpful discussions and criticisms of this project. We thank Dr. Ian Trowbridge for providing anti-Tf receptor antibody 42/6. This work was supported by U.S. Public Health Service Grant CA37393 from the National Cancer Institute.

1. Silverberg, E., Boring, C. C. & Squires, T. S. (1990) *Ca Cancer J. Clin.* **40**, 9–26.
2. Carter, H. B. & Coffey, D. S. (1990) *Prostate* **16**, 39–48.
3. Berrettoni, B. A. & Carter, J. R. (1986) *J. Bone J. Surg. Am.* **68A**, 308–312.
4. Jacobs, S. C. (1983) *Urology* **21**, 337–344.
5. Batson, O. V. (1942) *Ann. Surg.* **112**, 138–139.
6. Batson, O. V. (1942) *Ann. Intern. Med.* **16**, 38–46.
7. Chackal-Roy, M., Niemeyer, C., Moore, M. & Zetter, B. R. (1989) *J. Clin. Invest.* **84**, 43–50.
8. Kaighn, M. E., Narayan, K. S., Ohnuki, Y. & Lechner, J. F. (1979) *Invest. Urol.* **17**, 16–23.
9. Stone, K. R., Mickey, D. D., Wunderli, H., Mickey, G. H. & Paulson, D. F. (1978) *Int. J. Cancer* **21**, 274–281.
10. Mossman, T. (1983) *J. Immunol. Methods* **65**, 55–63.
11. Carmichael, J., DeGraff, W. G., Gazdar, A. F., Minna, J. D. & Mitchell, J. B. (1987) *Cancer Res.* **47**, 936–942.
12. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
13. Sammons, D. W., Adams, L. D. & Nishikawa, E. E. (1981) *Electrophoresis* **2**, 135–141.
14. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4350–4354.
15. Werner, P. A. M., Galbraith, R. M. & Arnaud, P. (1983) *Arch. Biochem. Biophys.* **226**, 393–398.
16. van Eijk, H. G. & van Noort, W. L. (1976) *J. Clin. Chem. Clin. Biochem.* **14**, 475–478.
17. De Jong, G., van Dijk, J. P. & van Eijk, H. G. (1990) *Clin. Chim. Acta* **190**, 1–46.
18. Cavanaugh, P. G. & Nicolson, G. L. (1989) *Cancer Res.* **49**, 3928–3933.
19. Trowbridge, I. S. & Lopez, F. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 1175–1179.
20. Taetle, R. & Honeysett, J. M. (1987) *Cancer Res.* **47**, 2040–2044.
21. Zetter, B. R. (1990) *N. Engl. J. Med.* **322**, 605–612.
22. May, W. S. & Cuatrecasas, P. (1985) *J. Membr. Biol.* **88**, 205–215.
23. Huebers, H. A. & Finch, C. A. (1987) *Physiol. Rev.* **67**, 520–582.
24. Grayhack, J. T., Lee, C., Oliver, L., Schaeffer, A. J. & Wendel, E. F. (1980) *Prostate* **1**, 227–237.
25. Barnes, D. & Sato, G. (1980) *Cell* **22**, 649–655.
26. Hayashi, O., Noguchi, S. & Oyasu, R. (1987) *Cancer Res.* **47**, 4560–4564.
27. Riss, T. L. & Sirbasku, D. A. (1987) *In Vitro Cell Dev. Biol.* **23**, 842–849.
28. Yoshinari, K., Yuasa, K., Iga, F. & Mimura, A. (1989) *Biochim. Biophys. Acta* **1010**, 28–34.
29. Taetle, R. (1990) *Hematology* **18**, 360–365.
30. Gatter, K. C., Brown, G., Trowbridge, I. S., Woolston, F. F. & Mason, D. Y. (1983) *J. Clin. Pathol.* **36**, 539–545.
31. Faulk, W. P., Hsi, B. L. & Stevens, P. J. (1980) *Lancet* **ii**, 390–392.
32. Keer, W. P., Koslowski, J. M., Tsai, Y. C., Lee, C., McEwan, R. N. & Grayhack, J. T. (1990) *J. Urol.* **143**, 381–385.
33. Cavanaugh, P. G. & Nicolson, G. L. (1991) *J. Cell. Biochem.* **47**, 1–11.
34. Batra, J. K., Fitzgerald, D. J., Chaudhary, V. K. & Pastan, I. (1991) *Mol. Cell. Biol.* **11**, 2200–2205.